Testing the Effect of Pharmaceutical Residues on the Cattle Dung Ecosystem

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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Zürich 2011

Contents

GENERAL INTRODUCTION

Vertebrate dung is a resource for many different species. Bacteria, fungi, nematodes, earthworms, mites and insects live in this ephemeral habitat. Some taxa visit the dung to forage or for shelter. Others depend on cattle dung for their development; they cannot survive without it and the species community associated with it. Many of the dung-inhabiting species have a strong preference for dung from certain groups of vertebrates (e.g. Gordon 1983). In many parts of the world the dung of cattle is a common kind of dung resource. The affiliated ecosystem is probably the best-explored dung ecosystem in general (e.g. Hammer 1941, Mohr 1943, Laurence 1954, Legner & Olton 1970, Merritt 1976, Holter 1979, Cervenka & Moon 1991, Mendes & Linhares 2002). Probably because they are the largest and most conspicuous organisms, insects have been the focus of most studies. For North America, more than 450 species (Blume 1985) and for Great Britain at least 275 species (Skidmore 1991) are known to be associated with cattle dung.

Beetles, flies and wasps are the most speciose insect taxa of cattle dung. While all dung-associated wasps exclusively are parasitoids of other insects (e.g. Figg et al. 1983), the other two groups include more diverse developmental strategies. Beetles can be parasitoids, predators or coprophages. Coprophagous species do not necessarily feed on undigested material in the dung; they can also live on excreted cattle gut fauna, fungal matter, or anything else not easily identified as prey. The larvae of some rove beetles (Staphylinidae) develop as parasitoids inside the larvae of certain fly species (e.g. Maus et al. 1998). Most Staphylinidae are predatory both as larvae and as adults. Two other groups of dung-inhabiting beetles with predatory larvae are the Histeridae (e.g. Hafez 1939) and the Hydrophilidae (e.g. Mohr 1943). The dung-dwelling Scarabaeidae make up the coprophagous guild of the beetles. Many species in this taxon have developed parental care, removing large parts of a dung pat quickly and burying them at a distance from the pat (Hanski & Cambefort 1991). Most Scarabaeidae larvae feed on undigested plant material and possess fermentation chambers containing symbionts that metabolize the plant fibers.

In contrast, all adult beetles feed on liquid material (Halffter & Matthews 1966). Impressive evidence for the importance of dung beetles for the decomposition of cattle dung comes from Australia. Dung of introduced cattle degraded extremely slowly and, with the numbers of cattle ranging in the millions, deteriorated the quality of cattle pastures. After realizing that insects abundant in dung in other regions of the world could be a solution to the problem (Bornemissza 1960), hundreds of thousands of scarabaeid dung beetles were released comprising 43 species. 23 of them became eventually established and significantly improved the situation (see Edwards 2007).

Like the beetles, also the dung-breeding flies have different feeding strategies (see Table 1, chapter 4). Some fly larvae are predatory. But in contrast to most beetle species, many predatory fly larvae change their strategy during larval development. While they are saprophagous during the first larval stages, they become predators in the last stage. Furthermore, some usually predatory species can complete their development on dung alone. The majority of fly species is probably coprophagous in the larval stage.

In addition to predation (see also Valiela 1969, Macqueen & Beirne 1975, Fay & Doube 1983, Roth et al. 1983), competition plays an important role in the dung ecosystem (e.g. Legner 1978, Tyndale-Biscoe et al. 1981, Roth et al. 1983, Sigurjonsdottir 1984, Ridsdill-Smith et al. 1987, Hirschberger & Degro 1996, Hirschberger 1999). Both intraspecific competition (Amano 1983) and interspecific competition (Fay & Doube 1983) have been demonstrated. But a lot of species also benefit from the presence of others, the most prominent example probably being tunnel-digging dung beetles (e.g. Valiela 1974). Their tunnels aerate the dung pat and thus increase oxygen supply for numerous other members of the community. Some fly species use these tunnels to enter deep into the pat to deposit their eggs, and predatory beetles to attack their prey.

To improve the output of livestock breeding many different pharmaceuticals are used. Antiparasitics are one important group. Some of them are active against a broad range of organisms, for example the avermectins, which are

effective against almost all parasitic nematodes and arthropods (http://www.vetpharm.uzh.ch/WIR/00007028/8867__F.htm). The parasitic arthropods comprise a large number of species of mites (Acari), fleas (Siphonaptera), flies (Diptera) and lice (Phthiraptera). Furthermore, many species of beetles (Coleoptera), wasps (Hymenoptera) and non-parasitic flies were shown to be sensitive to avermectins (see below). Many other arthropods, for example *Gammarus* (Amphipoda; Grant & Biggs 1998), Daphniidae (Cladocera; e.g. Lopes et al. 2009) and *Lernaea* (Copepoda; Hyland & Adams 1987), are also highly sensitive. Avermectins are not effective and hence not applied against plathelminth parasites and, obviously, vertebrates. Also, gastropods (e.g. Grant & Briggs 1998) and annelids (e.g. Svendsen 2002) seem to be much less sensitive. The pattern related to the sensitivity towards the avermectins seems to reflect the phylogenetic hypothesis of Aguinaldo et al. (1997). They unexpectedly postulated that nematodes, together with a few other taxa, form a monopyletic group with the arthropods named Ecdysozoa. One could therefore speculate that the physiological mechanism for increased sensitivity towards avermectins arose in the Ecdysozoa.

Avermectins are produced by the micro-organism *Streptomyces avermitilis* (Burg et al. 1979). The most important avermectin is ivermectin, which is a semisysnthetic substance obtained through the chemical modification of the original metabolite of *Streptomyces avermitilis* (e.g. Arlt & Bonse 2000). One of its mechanisms of action is the inhibition of neurotransmission (e.g. Yates & Wolstenholme 2004). The ion-channels involved in this process do not exist in vertebrates. Therefore, and for its efficacy, ivermectin seems perfectly predisposed for application in livestock breeding. Thus it is frequently used, not only in case of acute parasitization, but also prophylactically in many livestock animals and even in humans (e.g. Taylor 1989). Excretion occurs through the feces (Chiu & Lu 1989) and the excreted compound is largely unaltered ivermectin (Halley et al. 1989, Laffont et al. 2001, Gokbulut et al. 2005). At first this was considered a positive side effect of ivermectin treatment, as the larvae of blood feeding and disease transmitting flies breeding in the dung are eradicated from ivermectin-containing feces (e.g.

Schmidt & Kunz 1980, Miller et al. 1981, Schmidt 1983, Drummond 1985). Only several years after ivermectin had been in use, concerns over negative side effects were raised for the first time (Wall & Strong 1987). Many members of the dung breeding community have been shown to be sensitive to ivermectin (e.g. Miller et al. 1981, Schmidt 1983, Ridsdill-Smith 1988, Clarke & Ridsdill-Smith 1990, Cook 1991, Sommer 1992, Barth et al. 1994, Gover & Strong 1995, Strong et al. 1996, Floate 1998, Errouissi et al. 2001, Iwasa et al. 2005, Römbke et al. 2009, 2010). Besides the reduction of nematode and arthropod species in the dung community, the most severe problem appears to be the reduced degradation of dung from treated animals. While many studies show that the breakdown of ivermectin-containing dung is reduced (Wall & Strong 1987, Madsen 1988, 1990, Herd et al. 1993, Strong et al. 1996, Floate 1998, Dadour et al. 1999, Sommer 2002, Iglesias 2006, Römbke 2010), some studies do not find a significant effect (Schmidt 1983, Jacobs et al. 1988, Barth et al. 1993, Wratten et al. 1993, Barth et al. 1994), and one study concludes that degradation is accelerated with ivermectin (McKeand et al. 1988). One possible explanation for these contradicting results could be the varying composition of the dung community in different seasons and regions. Ivermectin-sensitive taxa do not always and everywhere play a major role in dung breakdown. Kaneda (2006) conducted a study in a region where mainly earthworms are responsible for dung degradation. Earthworms are relatively insensitive to ivermectin and dung breakdown was not retarded. In the (unlikely) theoretical case where ivermectin-sensitive taxa are feeding on the organisms mainly responsible for dung breakdown, it would even be possible that degradation is accelerated after ivermectin treatment. However, the general pattern seems to be that ivermectin-sensitive taxa are also mainly responsible for dung breakdown and therefore ivermectin-treatment usually slows down dung degradation.

The first step in assessing the non-target effects of pharmaceutical residues typically is the testing of a single species in a laboratory bioassay. But even when knowing the effect of a toxic substance on all the different single species comprising an ecosystem, it remains difficult to predict the effect on the whole ecosystem. As described above, interactions within the dung

ecosystem are numerous and complex, and reduction or removal of species in general has severe consequences for any ecosystem (Pimm 1980). Competition, predation and parasitism strongly constrain every species. These biotic factors interact with the pharmaceutical residues. In the most extreme case such residues can cause the extinction of a species. Subsequently, all its competitors are released from a constraint. If the effect of competition is stronger than that of the pharmaceutical, the latter can have a net positive effect on the remaining species leading to an increase of its population size. Of course, a species does not only interact with a single other species; several other competitors, predators or parasites will occur. Consequently, all these biotic interactions need to be taken into account to predict the reaction of one particular species to pharmaceutical residues. Furthermore, the residues might be affected by other abiotic factors such as light irradiation, temperature or humidity. Ivermectin, for example, is highly sensitive to UV-light (Reinemeyer & Courtney 2001) and should therefore be more persistent and effective in shaded environments. Summing up, it is indispensable to conduct field studies to assess the effect of pharmaceutical residues. These field studies should not be restricted to one locality, but include different environments to account for an interaction.

In this study, we first set up theoretical recommendations on how to conduct field studies to test the effect of pharmaceutical residues in the dung of livestock (Chapter 1). To establish standardized methodology in laboratory testing of pharmaceutical residues, we also assessed the impact of ivermectin on a single species in seven different laboratories (Chapter 2). We then assessed the effect of ivermectin on the dung insect community in a large-scale landscape field study, including 25 different localities in Switzerland, three seasons and two years (Chapter 3). This was done by comparing the impact on both biodiversity as a whole, as well as on different member species and ecological groupings (Chapter 4). While in the latter two studies, only one, intermediate concentration of ivermectin was evaluated, in a second study, we assessed the effect of six different concentrations of ivermectin at a single locality (Chapter 5).

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How to test non-target effects of veterinary pharmaceutical residues in livestock dung in the field

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published in: Integrated Environmental Assessment and Management

ABSTRACT

To register veterinary medicinal products (VMPs) as parasiticides on pastured animals, legislation in the European Union requires an environmental risk assessment to test the potential nontarget effects of fecal residues on dung-dwelling organisms. Products with adverse effects in single-species laboratory tests require further, higher-tier testing to assess the extent of these effects on entire communities of dung-dwelling organisms under more realistic field or semifield conditions. Currently, there are no documents specifically written to assist researchers in conducting higher-tier tests or to assist regulators in interpreting the results of such tests in an appropriate context. Here we provide such a document, written by members of the SETAC Advisory Group DOTTS (DungOrganismToxicityTestingStandardization) with research experience on dung fauna in central and southern Europe, Canada, Australia, and South Africa. This document briefly reviews the organisms that make up the dung community and their role in dung degradation, identifies key considerations in the design and interpretation of experimental studies, and makes recommendations on how to proceed.

INTRODUCTION

The Veterinary International Cooperative on Harmonization (VICH) is a trilateral program to harmonize technical requirements for the registration of veterinary medicinal products (VMPs) in the United States, Europe, and Japan (VICH 2004). Toward this end, the European Union (EU) now requires an environmental risk assessment of VMPs (EU 2009) that, in part, tests whether fecally excreted VMPs have nontarget effects on dung-dwelling organisms. The EU also requires that products with adverse effects in single-

species laboratory tests undergo further, higher-tier testing to assess the extent of these effects on the entire dung organism community under more realistic field or semifield conditions. Thus, it is very likely that many more higher-tier tests will be performed in the near future in different geographic areas characterized by various climate and dung faunas, and greater importance will be placed upon their results.

Numerous studies have assessed the effects of VMPs on dung organisms, both in the laboratory and in the field, using different methods (reviews by Floate et al. 2005; Lumaret and Errouissi 2002; Wardhaugh 2005; also Floate 2007; Floate et al. 2008; Hempel et al. 2006; Iwasa et al. 2007, 2008; Kryger et al. 2005, 2007; Lumaret et al. 2007; Römbke et al. 2007, 2009; Suarez et al. 2009; Webb et al. 2007, 2010). Collectively, these studies support the following conclusions: 1) there has been a strong bias for research on endectocides, primarily ivermectin but also doramectin, eprinomectin, and moxidectin; 2) most of the research studies have been performed in Europe, Canada, and Australia, and to a lesser extent in Japan, South Africa, South America, and the United States; 3) the lethal effects of residues on species of dung-breeding flies and beetles in cattle dung are most often measured; 4) because insect activity can accelerate dung pat degradation, the effect of residues on the rate of dung decomposition also is often measured in field-based studies; 5) the lack of standard test methods and reporting protocols largely prevents direct comparisons among studies; and 6) the interpretation of results is generally hampered and easily confounded by a lack of knowledge about the local biological aspects of the study system, i.e., the dung pat and its associated organisms, which may vary considerably among geographic regions.

With the expectation that legislation will require the standardization of higher-tier tests to assess the effects of VMPs on dung fauna, the German Federal Environment Agency (Umweltbundesamt) hosted 3 workshops (2007–2009). This paper is a result of those workshops and is intended to serve as a how-to guide for both regulators and researchers. We first summarize general aspects of the dung pat community and the biotic and abiotic factors that influence dung degradation. This information is needed to provide an appropriate context for subsequent discussion. We then present

key considerations for the design and interpretation of higher-tier tests. Many previous studies have overlooked one or more of these points. We conclude with a set of recommendations. We emphasize that our intent is not to provide a comprehensive review of the literature on dung fauna and associated testing. Rather, we hope to focus discussion on key considerations to facilitate future development of standardized field-based tests. Neither is it our intention to ignore the role of modeling, which may provide a method to supplement or avoid the need for higher-tier tests (Boxall et al. 2007; Vale and Grant 2002; Wardhaugh et al. 2001c). In the current paper, however, it is assumed that the decision for higher-tier testing already has been made.

The dung pat community

From time of deposition to total degradation, a dung pat may contain several dozen species of coprophilous arthropods (insects and mites) exceeding 1000 individuals (Laurence 1954; Mohr 1943; see Table 1). For Britain, Skidmore (1991) listed 275, 213, and 110 species of insects in dung of cattle, horses, and sheep, respectively. For North America, Blume (1985) listed over 450 species of insects associated with cattle dung. In tropical ecosystems, the number of coprophilous insect species is even higher, especially in Africa, where a very species-rich dung beetle community has evolved in response to the large number of herbivorous species. Bernon (1981) recorded 742 to 1585 beetles per pat, representing 161 species, colonizing fresh cattle dung pats over a 24-h period in central South Africa during summer, as well as numerous mites, flies, and other arthropods. Doube (1986) recorded 321 species of beetles, over 20 species of flies, over 100 species of mites, and a few species of ants associated with dung of animals found in the Hluhluwe Game Reserve in coastal Natal, South Africa. The vast majority of dung-associated taxa are either innocuous or desired either as natural enemies of pest flies or to accelerate dung degradation. Worldwide, only a few of these taxa are considered pest species; e.g., horn fly (*Haematobia irritans irritans* L.), buffalo fly (*H. i. exigua* De Meijere), face fly (*Musca autumnalis* De Geer), bush fly (*Musca vetustissima* Walker), and stable fly (*Stomoxys calcitrans* L.).

Dung pat communities comprise arthropod guilds that are characterized

by differences in diet (Figure 1). The larvae of dung-feeding flies, which include most species of coprophilous flies, feed on microorganisms. Early-instar larvae of mixed-diet flies feed on microorganisms and then switch, usually in the last instar, to feed on insects. Larvae of predatory flies feed only on insects. Dung-feeding beetles (mainly Scarabaeidae) feed solely or primarily on dung. Adults within this guild are filter-feeders (Holter 2000) and probably feed mostly on the microorganisms present in the fluid component of fresh dung (Aschenborn et al. 1989). In contrast, larvae of dung-feeding beetles ingest undigested plant fiber from which nutrients are extracted through the action of symbiotic cellulose-digesting bacteria housed in the larval hindgut (Terra 1990). Predatory beetles (mainly Staphylinidae) feed on other insects, particularly the eggs and larvae of flies. Fungivorous beetles colonize pats at later stages of decomposition and feed on fungal hyphae and spores. Wasps associated with dung are mainly parasitoids of dung-breeding flies. In addition, arthropods that arrive to colonize fresh dung may carry mites, nematodes, bacteria, and fungal spores that quickly increase in number once introduced to the pat. Predatory mites feed on nematodes or immature insects. The growth of bacteria and fungi accelerate dung degradation.

Colonization of fresh dung usually starts with flies and winged beetles, some of which arrive immediately after deposition and feed, mate, and lay eggs that produce a new generation in about 2 to 3 weeks. Fly numbers rapidly decline after a few hours, by which time crust formation on the pat has reduced the release of volatile attractants. Most dung-feeding beetles arrive shortly thereafter to feed and oviposit, with colonization peaking usually within the first week after deposition. Dung-feeding beetles form 3 general groups termed dwellers, tunnelers, and rollers. Dwellers complete egg-to-adult development within the pat or at the interface between the pat and the soil surface and are the dominant group in temperate climates. Adult tunnelers remove from the fresh pat dung that is buried in more or less vertical tunnels that may extend 10cm or more into the soil. This dung provides food for larvae that hatch from eggs laid in the buried manure. Rollers have the same nesting behavior as tunnelers, but dung removed from the pat is first formed into balls that are rolled some distance from the pat prior to burial. Tunnelers and rollers tend to dominate in subtropical and tropical climates. Egg-to-adult

development time of dung-feeding beetles may be from weeks to months. Parasitic wasps, mites, and predaceous beetles arrive concurrently with the flies and dung-feeding beetles and may either oviposit or feed on immature insects developing in the dung pat. There is very little additional colonization of dung by coprophilous insects 2 to 3 weeks after deposition, but adult beetles of some species may remain within the dung for more than 2 weeks after arrival.

The final colonization phase occurs with the breakdown of the interface between the dung and the soil surface. This process provides soil-dwelling organisms (e.g., earthworms, enchytraeids, bacteria) access to complete the breakdown of the dung. Depending on geographic region and season, earthworms may play a greater role in dung degradation than dung-dwelling insects (Holter 1979). During this latter phase, decomposing pats may be visited by taxa that are searching for food or shelter, or that are attracted to rich organic soils and rotting vegetation. Such taxa may include centipedes (Chilopoda), woodlice (Isopoda), millipedes (Diplopoda), harvestmen (Opiliones), spiders (Araneae), earwigs (Dermaptera), springtails (Collembola), termites (Isoptera), ants (Formicidae), click beetles (Elateridae), ground beetles (Carabidae), and bugs (Hemiptera). These incidental species are not normally considered to be part of the dung pat community, because they do not rely on dung as a breeding substrate.

Because insect activity accelerates dung degradation, rapid removal of dung from the pasture surface often is used, incorrectly, as an indicator of the health of the dung insect community. Degradation reflects the interaction of a complex of biotic and abiotic factors (Merritt and Anderson 1977). Livestock stocking rates affect the likelihood of pats being disrupted by trampling. Birds foraging for insects or seeds can quickly fragment pats. Shade reduces the rate of pat desiccation, which makes the pat attractive to insect colonists for a longer period. Heavy rainfall quickly causes the dissolution of fresh pats. Warm or wet conditions usually initiate peak insect activity, which generally is lowest when conditions are cold or dry. The moisture and fiber content of the animal’s diet affects the compactness of the dung and its resistance to degradation. Tropical agroecosystems often are characterized by large dung beetle species (rollers, tunnelers) that can fragment and bury dung pats within

hours of deposition (Cambefort and Hanski 1991). North temperate regions are more often characterized by small species (dwellers), which do not bury dung but only slowly degrade the pat during a period of weeks through the feeding activity of their larvae (Cambefort and Hanski 1991). Depending on this complex of factors, complete incorporation of a healthy dung pat into the soil may take from weeks to years (Merritt and Anderson 1977).

CONSIDERATIONS

Whichever VMP is to be tested, the researcher should report the name and concentration of the active ingredient, its formulation, the method of application, the number and type of animals being treated, and the dose (per kg body wt) of the applied treatment. For those organizations requiring them, animal care protocols should be submitted to and approved by the local animal care committee prior to starting animal-based research. In addition, it may be desirable to quantify the concentration of residue in fresh dung deposited at various times posttreatment or in dung as it ages after deposition. Different methods are available (Bousquet-Melou et al. 2004; Floate et al. 1997; Lumaret et al. 2005; Vale et al. 2004).

Single-species assays performed in laboratories, such as the standardized dung fly test (OECD 2008) and dung beetle test (OECD 2009), provide an inexpensive and effective method to test the toxicity of VMP residues in dung (Floate et al. 2001; Hempel et al. 2006; Kryger et al. 2006, 2007; Lumaret et al. 2007), but these are simplistic. Results from the test species provide a basis for a conservative estimate of possible environmental toxicity but almost certainly will not predict the toxicity of residues to all nontest species. Furthermore, they do not take into account the complex interactions that occur among the diverse taxa in naturally colonized dung pats, and they may not accurately assess residue toxicity when the product is used as proposed. Higher-tier tests using field-colonized dung may be useful to assess more accurately the nontarget effects of residues under more realistic conditions and on a broader range of coprophilous species. Field-based tests require consideration of one or more of the following.

Protection goals

The intent of regulation is to protect the structure (biodiversity) and function (ecosystem services) of the dung pat community from potential nontarget effects of VMPs. Structure is defined as the number and relative abundance of species. Function can be measured in a crude way as the rate of dung decomposition, which integrates the activities for all members of the community. Protecting structure can be expected to protect function (Calow 1996). Therefore, it is most efficient to measure the effect of residues on the biodiversity of the dung community, although measuring dung degradation may still be desirable and is sometimes easier.

Dung quality

Dung of different livestock species will attract different numbers and species of coprophilous arthropods. For example, dung from sheep versus cattle may attract a subset of the same species, but their relative abundance will differ. Thus, results reporting the fecal toxicity of VMP residues are not directly transferable among livestock species.

When livestock species is held constant, variation in dung quality (moisture content, fiber, pH) still may confound the detection of nontarget effects associated with fecal residues (Barth et al. 1995; Cook et al. 1996). This may be of concern if fresh dung is collected from 2 or more groups of animals on different diets or from 1 group of animals whose diet has significantly changed during the period of dung collection. For example, it is common for dung of pastured animals to vary in fiber and moisture content during the course of the grazing season in response to seasonal changes in pasture forage.

Variation in dung quality unrelated to VMPs can be minimized with different methods. One method is to collect fresh dung for the experiment on the same date from untreated animals and from one or more groups of treated animals (Holter et al. 1993). By applying treatments on different dates prior to the date of collection, it is possible to compare, for example, the toxicity of fecal residues in fresh dung from animals treated 1 and 2 weeks previously to that of a control group of untreated animals (Method 1 in Figure 2). This method allows for changes in diet that are simultaneous across groups but

also introduces intergroup variation unrelated to VMP residues. The significance of the latter can be assessed by comparing the dung from control and treatment groups prior to treatment.

A second method involves the collection of fresh dung for the experiment from one group of animals before and at various times posttreatment (Method 2 in Figure 2). The dung is frozen until collections are completed, then thawed to permit simultaneous comparisons among different collections of dung; e.g., dung collected pretreatment versus dung collected 1 and 2 weeks posttreatment (Floate 1998b; Floate et al. 2002, 2008). This method requires fewer animals than the first method and eliminates intergroup variation as a potential confounding factor. However, the diet and holding conditions of the animals must be held constant throughout the period of dung collection. Freezing the dung does not appear to affect the toxicity of endectocide residues (K Floate and J-P Lumaret, personal observations). Variations of these 2 methods have been described by Kryger et al. (2005, 2006, 2007) and Wardhaugh et al. (2001c).

Unwanted VMP residues in control dung

Parasiticide treatments applied to livestock months previously may result in the presence of residues in dung of the untreated control group and thus confound the interpretation of results. Topical application of some VMPs may result in fecal excretion of insecticidal residues for extended periods; e.g., over 16 weeks for doramectin (Floate et al. 2008) and over 12 weeks for ivermectin (Floate 1998b). Animals treated with sustained-release devices may excrete insecticidal residues for periods exceeding 100 to 150 d (Errouissi et al. 2001; Wardhaugh et al. 2001a; Wardhaugh et al. 2001b). Furthermore, grooming among animals can result in the transfer of topically applied VMPs. Thus, VMP residues may be present in dung of untreated cattle housed with treated cattle (Bousquet-Melou et al. 2004).

Dung from treated animals versus spiked dung

Greatest realism is obtained by using dung from animals treated with the compound in a formulated product. Such dung may include residues of both the parent compound and the metabolites formed during passage of the

parent compound through the animal. Its use also accounts for potential changes in the gut flora of the animal associated with the treatment, which in turn may affect the quality of the dung for coprophilous organisms. Use of treated animals, however, requires access to animals held under controlled conditions for potentially extended periods, and legislated risk assessments may require levels of fecal residues to be quantified. Both of these requirements can significantly increase the cost of the study. Alternatively, dung can be collected from untreated animals at one point in time and spiked with known concentrations of the test compound. This allows for greater control in testing the compound across a range of concentrations and eliminates both variation in dung quality and the need for long-term access to animals. However, the method excludes consideration of metabolites and potential treatment effects on gut flora and may be impractical in some cases. The more watery consistency of cattle dung makes it much more amenable to being spiked than dung from sheep or horses.

Natural versus artificially formed pats

The effects of VMPs can be studied in naturally deposited pats or pats that have been artificially formed. The former introduces variation associated with factors that may include but are not limited to the time of deposition, location (shaded vs. unshaded), substrate (bare soil vs. vegetation), weather, pat size, number of replicate pats per treatment, and distance between pats. Furthermore, animals typically will have to be excluded from pastures after deposition of the pats to prevent the latter from being trampled. Conversely, dung from naturally deposited pats can be combined, thoroughly mixed to reduce potential variation in dung quality across pats, and then used to make artificially formed pats. Artificially formed pats can be standardized for size, spacing, time and duration of exposure, and number of replicate pats per treatment. There also is greater flexibility in the siting of experiments (in pastures vs. adjacent to pastures) and in setting the number of replicate pats. Accordingly, intrapat variation is reduced so that data from artificially formed pats are more likely to detect potential treatment effects than data from naturally deposited pats.

Season and duration of study

Insect activity and abundance generally increase with temperature or rainfall. Thus, studies performed in unseasonably cool or dry conditions are unlikely to provide a satisfactory assessment of the effects of pesticide residues on insect abundance or pat dispersal. This is true for both tropical and temperate habitats.

The duration of the study may affect the quality of the results. Local assemblages of dung beetles may include univoltine, multivoltine, and opportunist species. For univoltine species, diapause is obligatory, such that breeding insects are active in the warmest or wettest parts of the year, but with only a single annual generation. For multivoltine species, diapause may be facultative, intervening prior to the onset of unfavorable seasonal conditions (e.g., a cold winter or a hot, arid summer), yet allowing one or more annual generations. For opportunist species, breeding is continuous with favorable temperature and rainfall conditions. Thus, the effect of drug residues on subsequent generations may not be apparent for univoltine species for one or more years versus 1 to 2 months for multivoltine or opportunist species.

The nontarget effect of fecal residues can be assessed by recording the number of insects developing in dung pats that have been exposed in the field and then held in cages for insect emergence (Floate 1998b; Floate et al. 2002, 2008). Pats exposed for a short period may exclude some species that are attracted to old dung; e.g., some species of Aphodius beetles. Pats exposed for a long period increase the likelihood that fast-developing species, such as flies, will complete development and emerge before pats are placed in the cages.

Sample replication

Statistical power is needed to provide confidence that negative results reflect a true lack of effect and are not an artifact of small sample numbers, and also to offset the typically large variation in data obtained. Greater confidence is achieved by using a large number of replicate dung pats per treatment. When assessing the insecticidal activity of dung residues, increased replication will increase the number of insects to be sorted,

counted, and identified. Because several hundred insects representing several dozen species may emerge from one dung pat, tens of thousands of insects may be recovered in one experiment. Hence, the need for statistical power must be balanced against the cost and time to process samples. In general, the rarer a species is, the more dung pats have to be sampled to achieve meaningful sample sizes for this species.

Potentially, a subset of species could be used as bioindicators of a larger taxonomic group or guild; e.g., dung-feeding flies. Alternatively, species could be combined into higher taxonomic groupings for analyses (but see below under Level of taxonomic resolution). Insect samples also can be collected from a large number of replicate dung pats, with only a subset being processed for analyses. The remaining samples can be processed if results indicate a need for greater statistical power.

Because of the limitations of null hypothesis testing, even studies with high statistical power risk Type II errors (false negatives). This risk can be reduced by reporting effect size values (e.g., measures of central tendency in all treatments) and the confidence intervals around these measures (Nakagawa and Cuthill 2007), in addition to tests of significance. Consistent reporting of measures will greatly enhance the ability to compare results across studies.

Lethal effects

The toxicity of fecal residues can be assessed by comparing numbers of insects developing in dung with and without residues. Counts can be performed on immature stages (larvae and pupae) or on adult insects. Counts of immature flies usually can be obtained within 2 weeks postcolonization, varying with species' developmental time. Because of the much longer developmental times, counts of immature beetles may require weeks or months, particularly for species with a larval or pupal dormancy. Species identifications often are not possible with immature stages, for which counts also may underestimate egg-to-adult mortality. For example, fly larvae that survive exposure to residues may be unable to complete development to pupal or adult stages (Strong and James 1993). Counts of immatures also will exclude some taxa; e.g., parasitic wasps developing inside other insects or

species of dung-feeding beetles whose larvae develop away from the dung pat (tunnelers and rollers). Conversely, counts of adult insects require use of a cage to prevent their escape when they emerge from the pat. Counts of adults also require a period of time longer than that needed for counts of immatures of the same species. In addition, adults recovered in cages may have colonized the pat in the field (colonists) or developed from egg to adult in the pat (progeny of colonists). Because progeny are more likely than colonists to be affected by fecal residues, analyses limited to the former group are more likely to detect potential treatment effects (Floate 1998b).

The indirect effect of VMPs applied to livestock on populations of dung-breeding insects in the field can be assessed by using dung-baited pitfall traps to compare numbers of adult insects (usually beetles) recovered in pastures grazed by untreated versus treated cattle (Krüger and Scholtz 1998a, 1998b; Kryger et al. 2006; Webb et al. 2010). Dung used for all baits should come from untreated cattle, because fecal residues of VMP may affect trap captures (see next paragraph). Such traps are easily operated for long periods of time with renewal of baits and emptying of traps at regular intervals (e.g., weekly or twice weekly). However, the movement of beetles among adjacent pastures may mask all but severe reductions of insects associated with use of VMPs. Trapping for extended periods may risk depletion of local populations, particularly in the case of larger species of dung beetles.

Use of VMPs can affect colonization such that more or fewer insects may lay eggs in dung of treated livestock (Floate 1998a, 2007; Holter et al. 1993; Wardhaugh and Mahon 1991). The insecticidal activity of residues in such dung may be somewhat underestimated if it initially contains a larger starting population of insects relative to control dung and vice versa. Operation of paired pitfall traps baited with dung of treated versus untreated animals can be used to test for an effect of residue on colonization.

Sublethal effects

Sublethal concentrations of fecal residues potentially may prolong insect developmental time (Römbke et al. 2009), reduce the size and longevity of adults, reduce mating success, alter sex ratios, decrease fertility, and reduce lifetime fecundity. Measuring development time, body size or weight, and

altered sex ratios is relatively easy. Measuring adult longevity, mating success, lifetime fecundity, and fertility is more difficult. Sublethal effects may or may not have long-term consequences for future generations of the exposed insect and for the community as a whole. The ability of populations to recover from reductions associated with lethal or sublethal exposure to fecal residues presumably will vary with factors including fecundity, generation time, and number of generations per year.

Morphological measurements of random deviations from perfect symmetry (termed fluctuating asymmetry, or FA) may be a particularly sensitive bioindicator of an organism's exposure to sublethal concentrations of fecal residues. Insects developing in dung with doramectin or ivermectin residues have been reported to exhibit higher levels of FA than conspecifics developing in dung without residues (Strong and James 1993; Webb et al. 2007). However, application of FA assessments can be very time consuming, and their application as a bioindicator has not been supported in more detailed studies (Floate and Coghlin 2010; Floate and Fox 2000).

Taxonomic resolution

Species-level identifications provide the least ambiguity in the interpretation of results and permit direct comparisons with other studies for which the same species were present. Each species also provides a separate test of residue toxicity. However, species-level identifications are time consuming and require a certain level of expertise. Thus, species may be grouped into higher taxa (e.g., genus, family, order) or morphospecies (i.e., species merely looking similar morphologically) to expedite processing. Species also may be grouped into larger taxa for analyses when low recovery of individuals prevents analyses on a per-species basis. However, grouping sensitive with insensitive taxa will lead to systematic underestimates of residue toxicity (Floate 1998b; Floate et al. 2001). Furthermore, for example, the Coleoptera of one study may differ in the number, type, and biology of species making up the Coleoptera of a second study. Depending on circumstances, it may be desirable to identify some organisms to species and other organisms to genus or family.

Dung degradation

Loss of mass (organic matter) over time is the preferred method to estimate the rate of dung degradation. Measurements can be confounded by the incorporation of soil particles into the pat via the activity of insects and earthworms or by physical disruption caused by scavengers (e.g., rodents, birds, foxes, wild pigs) or livestock. The former can be addressed by carefully separating soil and dung particles before measuring the loss of organic matter. This can be facilitated by placing a barrier (e.g., root cloth, meshing) beneath the pat to maintain the interface between dung and substrate. Note, however, that, although use of a barrier can exclude earthworms, it also may interfere with the activity of dung-burying beetles and therefore reduce the natural rate of dung degradation. Pats can be protected from small vertebrates by using small cages or enclosures (Ward and Wilhelm 1994). Animals can be removed from pastures, or artificially formed pats can be located outside of pastures to avoid trampling by livestock. Dung degradation can be quantified by visual estimates, but this method may be highly inaccurate and fail to detect losses as high as 70% (Holter 1979).

Number of experiments

Within a local area, the number, type and abundance of taxa dwelling in the dung of a given animal species are affected by many factors, including soil type, vegetation, weather, season, and both number and proximity of grazing livestock. Thus, results of one experiment may not predict results of the same experiment performed at a nearby site in the same year or at the same site in a different year. Replicating experiments across sites and time increases confidence in the size and consistency of observed effects on dung fauna communities. Replicating experiments in regions that differ greatly in climate and associated dung faunas (e.g., Europe vs. Africa) provides an even stronger method for assessing the generality of observed effects. This can therefore best be regarded as an invitation to several researchers to coordinate their efforts and replicate the same experimental approaches in various places and at various times.

RECOMMENDATIONS

We do not recommend one experimental method to the exclusion of all others. This would serve only to limit the number of studies available to assess the toxicity of fecal residues. However, researchers should be fully aware of the limitations associated with whichever method they choose. Failure to do this, for example, may produce erroneous conclusions affecting product registration that ultimately may be very expensive; thus ‘‘. . . the costs of an incorrect decision are arguably much larger than the costs of testing, though not as immediate’’ (Chapman 2002). To facilitate optimization of field-based studies, we list a number of recommendations below. Many of these recommendations will be obvious to some readers and seemingly not worth stating. However, not all readers will be researchers, and those who are may not all be entomologists.

Preparation

1) Clearly identify the purpose of the study. Is the intent to test the effect of VMP residues on the function (rate of dung degradation) or the structure (species richness and abundance) of the dung pat community? Are the lethal or the sublethal effects of residues to be assessed? Are effects to be measured for all taxa or just a subset of taxa? Are effects to be assessed under a natural range of field conditions or one set of uniform conditions? Is the goal to assess the nontarget effects of a single VMP application to organisms that colonize dung of the treated livestock or is it to assess the long-term effect of regular VMP use to endemic populations of coprophilous organisms in pastures housing treated livestock? Clarifying the research question will aid in subsequent planning.

2) Review the appropriate literature on the test and related compounds and on coprophilous organisms endemic to the study area. This will identify, for example, methods previously used to measure the test parameters, the expected extent of effect, and seasonal patterns of insect activity.

3) Design the study to critically test the hypothesis. If the insecticidal activity of VMP residues is to be tested, set up the experiment during peak periods of dung-insect activity. To determine whether the test compound has a long period of fecal excretion, include treatments of dung from animals treated weeks or months previously. Use sufficient replication to ensure

statistical power, and estimate effect sizes in addition to testing the null hypotheses. Replicate the study (see 8 below).

Implementation

4) Collect dung from appropriate sources. Standardize the breed, gender, age, diet, and treatment history of livestock across treatments. Do not use animals whose treatment histories may confound results; e.g., animals treated with ivermectin in the preceding 6 months. Do not hold treated and untreated animals together if residues may be transferred via grooming. Mix dung thoroughly within 1 treatment if it comes from different animals.

5) Collect more dung than is needed for the experiment. Heavy rain or unusually cool temperatures may require the experiment to be set up a second time. Additional dung also will allow for a larger number of replicate dung pats in the experiment in the event that some pats are disturbed by birds and small mammals or are otherwise lost. Set up experiments in areas that support a large endemic population of coprophilous organisms; i.e., where livestock have been present for a period of time.

6) Minimize confounding factors. Standardize pat size. When comparing a control group to 2 or more treatment groups, randomize the location of groups within the site. When comparing a control group to 1 treatment group, use a paired design, i.e., pair each replicate control pat to a corresponding treatment pat. Place all treatments at the site at the same time; e.g., do not set out control pats in the morning and treatment pats in the afternoon. Consider the use of cages to minimize disturbance of pats by birds and small mammals.

7) Assess the effects of VMP residues on insects using the smallest feasible taxonomic unit, in order of preference: species > genus > family > suborder > order. Whereas use of species permits direct comparisons across experiments and studies, such comparisons using order are essentially meaningless.

8) Replicate the experiment to test the generality of observations. Experiments can be replicated across sites, seasons (e.g., spring vs. autumn), or years. Ideally, experiments would be replicated at 2 or more sites in each of 2 years or more. The number of replicates per experiment may

vary. If results are comparable, 2 replicates of an experiment may suffice. If results differ, a third replicate of the experiment is desirable.

Evaluation

9) Question a conclusion of no treatment effect if numbers of insects in control dung are unexpectedly low. In such cases, the statistical likelihood of detecting a treatment effect is reduced and may indicate that the experiment was set up at an inappropriate time of the year or that insecticidal residues were present in the control dung.

10) Do not conclude that residues are noninsecticidal if treatment does not affect dung degradation. Other factors might have masked the role of insects in dung degradation (e.g., precipitation), or insects may have played a relatively minor role in dung degradation.

11) Do not overextrapolate results. The effect of residues on flies does not predict the effect of residues on a given species of fly. The egg to larval mortality of insects exposed to residues does not predict egg to adult mortality of similarly exposed insects. Results of an experiment at one site may not predict the results of the same experiment at a different site.

SUMMARY

Field-testing of veterinary pharmaceuticals for nontarget effects on dung fauna can be an important step in the assessment of their environmental impact. Usually, protecting biodiversity (the number and relative abundance of species) means protecting function (the rate of dung decomposition and associated ecosystem functions) but not vice versa. To be able to interpret and compare results, it is necessary to have at least some ideas about the dung community and the biotic and abiotic factors that influence this complex system. The community in a single dung pat may consist of several hundred species, including detritivores, bacterivores, predators, and parasitoids, i.e., dissimilar organisms with different life histories. Depending on the biogeographic region, soil type, vegetation, weather, season, and dung quality, the composition of the community and its role in dung pat degradation may differ considerably.

When designing an experiment, it is important to be aware of some key

points. When comparing dung of treated and untreated livestock, differences in the 2 groups unrelated to the treatment can lead to differences in dung quality and consequently in the dung community. When comparing dung of 1 group of livestock before and after treatment, changes occurring during this period of time can also lead to community differences unrelated to treatment.

Dung containing residues of VMPs can be obtained from treated livestock or by spiking dung from untreated animals. When using spiked dung, only artificially formed dung pats can be examined, whereas with dung from treated animals, natural ones can also be used. After treatment, animals may excrete residues with their dung for extended periods. Several other factors also can lead to erroneous results, including experiments conducted in seasons with low organism abundance and activity and grouping of insensitive with sensitive taxa. Finally, most research has looked at lethal effects of VMP residues, but sublethal effects can be useful indicators of a product's toxicity.

ACKNOWLEDGMENTS

We thank the German Federal Environment Agency (Umweltbundesamt) for hosting a series of 3 workshops, which brought together the authors and identified the need for this document. We also thank all of the support staff and students who have helped over the years to acquire the practical experience upon which this document is based. R Jochmann thanks the Velux Foundation for funding his PhD.

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Table 1. A partial list of taxa associated with livestock dung.[1] Species composition varies with biogeographical region.

Taxon (common name[2])	**Taxon (common name)**
Coleoptera (beetles)	• Sarcophagidae (flesh flies)
• Clambidae (fringe-winged beetles)	• Scathophagidae (dung flies)
• Cryptophagidae (silken fungus beetles)	• Sepsidae (black scavenger flies)
• Lathridiidae (minute brown scavenger beetles)	• Sphaeroceridae (small dung flies)
	• Stratiomyidae (soldier flies)
• Pselaphidae (short-winged mold beetles)	• Syrphidae (hover flies)
• Ptiliidae (feather-winged beetles)	Nematocera
• Histeridae (hister beetles)	• Anisopodidae (window gnats)
• Hydrophilidae (water scavenger beetles)	• Cecidomyiidae (gall midges)
• Scarabaeidae (scarab beetles)	• Ceratopogonidae (biting midges, punkies, or no-see-ums)
- Aphodiinae (aphodian dung beetles)	
- Geotrupinae (earth-boring dung beetles)	• Chironomidae (midges)
- Scarabaeinae (dung beetles, tumble bugs)	• Mycetophilidae (fungus gnats)
• Staphylinidae (rove beetles)	• Psychodidae (moth flies)
Hymenoptera (wasps)	• Scatopsidae (minute black scavenger flies
• Braconidae	• Sciaridae (dark-winged fungus gnats)
• Diapriidae	• Tipulidae (crane flies)
• Eucoilidae	Collembola (springtails)
• Figitidae	Acari (mites)
• Ichneumonidae	• Eviphididae
• Mymaridae (fairyflies)	• Halolaelapidae
• Proctotrupidae	• Macrochelidae
• Pteromalidae	• Parasitidae
• Scelionidae	• Uropodidae
• Tiphiidae	Nematoda (roundworms)
Diptera (flies)	• Bunonematidae
Brachycera	• Diplogastridae
• Anthomyiidae (anthomyiid flies)	• Panagrolaimidae
• Calliphoridae (blow flies)	• Rhabditidae
• Dolichopodidae (long-legged flies)	• Tylopharyngidae
• Empididae	Annelida
• Muscidae (muscid flies)	• Enchytraeidae
• Phoridae (scuttle flies)	• Lumbricidae
	• Megascolecidae

[1] For more detailed lists, see (Blume 1985; Skidmore 1991); [2] Common names as per (Borror et al. 1989).

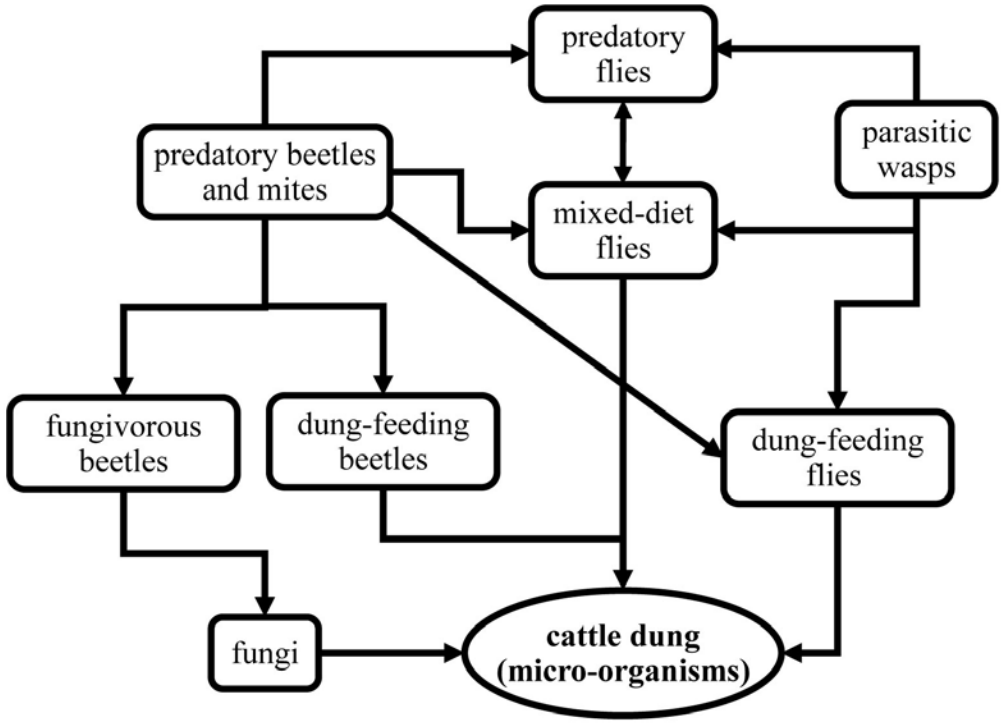

Figure 1. Type and interactions among arthropod groups common in cattle dung.

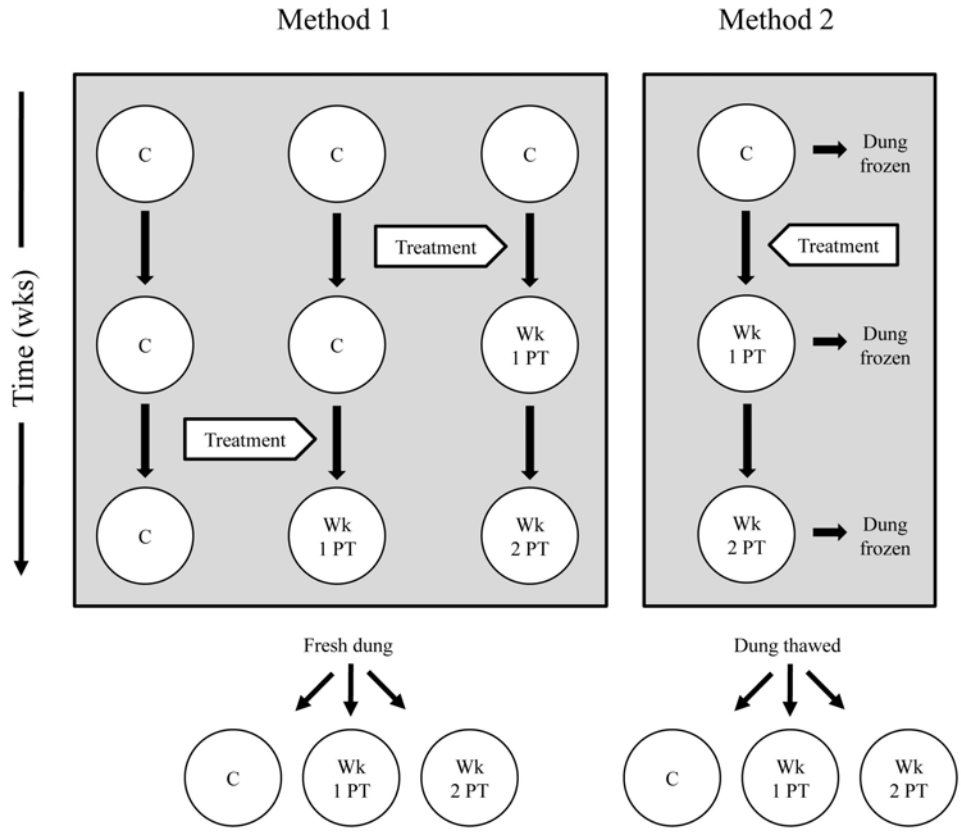

Figure 2. Comparison of methods for the collection of dung from untreated and treated animals. Method 1 requires use of more animals, but avoids the need to freeze dung prior to use. Method 2 uses fewer animals, but requires dung to be frozen prior to use. C = control, PT = post treatment, Wk = Week

Lethal and sublethal toxic effects of a test chemical (ivermectin) on the yellow dung fly S*cathophaga stercoraria* based on a standardized international ring test

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published in: Environmental Toxicology and Chemistry

ABSTRACT

A standardized bioassay using the yellow dung fly, *Scathophaga stercoraria* L. (Diptera: Scathophagidae), was developed to test the lethal and sublethal toxicity of parasiticide residues in livestock dung. The repeatability of the bioassay was assessed for the parasiticide ivermectin in 13 tests performed by seven laboratories in Germany, the United Kingdom, Switzerland, and Canada. Test results had an acceptable range of heterogeneity. The calculated median effective concentration for 50% (EC50) egg-to-adult mortality was 20.9 +/- 19.1 mg ivermectin/kg dung fresh weight (FW) (mean +/- standard deviation; range, 6.33–67.5 mg/kg). Mortality was not observed below a calculated no-observable-effect concentration (NOEC) of 8.1 +/- 7.7 mg/kg FW. However, prolonged development time (and, in a subset of tests, reduced body size) was observed above a calculated NOEC of 0.8 +/- 0.8 mg/kg FW. An oviposition site choice test revealed that yellow dung fly females do not discriminate among dung of different ivermectin concentrations. Thus, the yellow dung fly is suitably sensitive, and the methods are sufficiently repeatable, to support use of this standardized bioassay by the international community in the registration of new veterinary pharmaceuticals.

INTRODUCTION

Manufacturers of veterinary pharmaceuticals are required by regulatory agencies to demonstrate the quality, safety, and efficacy of new products as part of the registration process. The Veterinary International Co-operation on

Harmonization (VICH) exists to integrate the registration requirements across the United States, Japan, Europe, Canada, and Australia. Guidelines developed by VICH (2004) require an environmental risk assessment for residues of veterinary pharmaceuticals that are excreted in the dung of treated livestock. Such residues can adversely affect nontarget organisms breeding in dung that ultimately are responsible for the breakdown of dung, and these residues can potentially have larger effects in the pasture environment (for review, see Floate et al. 2005).

A draft bioassay was proposed by the Dung Organism Toxicity Testing Standardization (DOTTS) group in 2003, initiated by Jackie Hughes (Inveresk, Scotland, UK), who also provided the first draft of the test guideline, to test the insecticidal effects of contaminated fecal residues (OECD 2004a, http://www.oecd.org) using the yellow dung fly, *Scathophaga stercoraria* L. (Diptera: Scathophagidae). Selection of the species was based on its broad distribution in the north temperate zone (Blume 1985); its nonpest status and common occurrence in the dung of various livestock species (particularly cattle); its ease of rearing in laboratory culture, with a relatively short generation time (approximately six weeks); and its history of use in ecotoxicological studies (see, e.g., Madsen et al. 1988, Webb et al. 1991, Sommer et al. 1992, Strong & James 1992, Floate 1998, Floate 2007). Furthermore, extensive information is available regarding the evolutionary ecology, mating behavior, and reproductive strategies of *S. stercoraria* (for a summary, see Blanckenhorn 2009).

The current study tested the suitability of the DOTTS draft bioassay in an international ring test using the parasiticide ivermectin. Ring tests examine the following: Whether the bioassay is understandable and practical, if the proposed validity criteria can be met and reproduced among laboratories, whether a suitable reference substance can be recommended, and if the interlaboratory variability is sufficiently low, as required for and known from other such ecotoxicological tests OECD 2004a, OECD 2005). The present study reports the results of this ring test, which was performed as a cooperative effort by seven laboratories in Germany (n = 3 laboratories), Switzerland (n = 2), the United Kingdom (n = 1), and Canada (n = 1). It represents the final step in validating the DOTTS bioassay for use in

achieving VICH guidelines. The present study also included an oviposition site choice experiment testing whether yellow dung fly females discriminate against dung contaminated with ivermectin.

MATERIAL AND METHODS

Thirteen tests (S-1 to S-13) were performed among cooperating laboratories between 2003 and 2006 (Table 1) to examine the insecticidal activity of ivermectin in dung. In Europe, these tests were performed using technical ivermectin (Chemical Abstracts Service no. 70288-86-7), with a purity of 94% for ivermectin B1a and 2.8% for ivermectin B1b (supplied by Paul Cooper, Merial). In Canada, tests were performed using ivermectin in a topical formulation (IvomecH Pour-On for Cattle; Merial). Ivermectin is not soluble in water. Hence, it was first dissolved in acetone to obtain desired concentrations by serial dilution. The acetone/ivermectin solution then was mixed thoroughly into cattle dung and kept overnight at 20°C to allow evaporation of the solvent.

Each test comprised six (Canada) or seven (Europe) treatments, which included a water control, an acetone control, and four to five concentrations of ivermectin. Tests in Europe used ivermectin concentrations ranging from 0.67 to 65.7 mg/kg dung fresh weight (FW) and were selected based on results of two range-finding tests performed in the laboratories of IBACON (Rossdorf, Germany) and ECT (Flörsheim, Germany). Tests in Canada used ivermectin concentrations ranging from 0.25 to 250 mg/kg FW and were selected based on the results reported by Strong and James (1992).

With the exception of test S-4, which used flies caught wild near Yorkshire (UK), test flies originated from a laboratory stock of *S. stercoraria* maintained at the University of Zurich (Zurich, Switzerland) (for rearing methods, see Blanckenhorn et al. 2008). For the European laboratories, eggs typically were sent from Zurich for immediate testing. The Canadian laboratory established a colony using pupae received from Zurich, which then provided flies for tests.

Dung used in all tests was obtained from cattle that had not been treated with parasiticides for at least three months. Parasiticide-treated cattle may excrete residues for a period of three to four months, depending on the

product (Floate 1998, Floate et al. 2008). Dung was collected fresh and used immediately, or it was frozen at -20°C until used. The sole exception was dung used in test S-3, which was formulated (i.e., dried, ground, and rewetted). For a given test, all replicate treatments used dung collected from the same group of cattle at the same time. Dung typically had a moisture content of 85% and pH 7 to 8.

The experimental unit was a plastic vessel (volume, 500 ml; diameter, 7 cm; height, 13 cm) with an air-permeable lid to which was added 100 g of dung with 10 (Europe) or 25 (Canada) eggs. More than 2 g of dung per individual was sufficient to avoid larval competition (Amano 1983). Eggs from several females were counted onto a small piece of filter paper, which then was put on top of the dung in the vessel. This allowed the percentage of eggs hatched to be determined (Blanckenhorn et al. 2008) and, therefore, the adult mortality to be identified based on the starting number of larvae in the experimental unit.

The number of adult flies emerging in each vessel was recorded to document the lethal effect of ivermectin residues in dung. With the exception of test S-6, individual egg-to-adult development times were recorded to document sublethal effects. Sublethal effects additionally were assessed in tests S-8 and S-9 by recording the fresh body mass of emergent flies. Male and female *S. stercoraria* differ in development time and body size, with males being larger and slower to complete development (Blanckenhorn 1998, 2009). Hence, data were recorded separately for each sex. Tests were performed at 20 +/- 2°C (mean +/- range; room temperature) and were terminated 5d after the emergence of the last fly from the water control.

In accordance with Organization for Economic Co- operation and Development (OECD) Guideline 218 (OECD 2004b), values for the no-observable-effect concentration (NOEC) and for the concentration causing 50% egg-to-adult mortality (EC50) were determined using probit analysis with the program ToxRatH (2003). Analyses-of-variance tests were employed with the statistical program SPSSH (Ver 13, 2007) to assess the effect of treatment (and sex) on mortality, development time, and body mass using mean values for each container. Tests performed in Europe typically used five replicate vessels per treatment, whereas tests performed in Canada used 20

replicates per treatment. The larger number of replicates for the Canadian tests reflected greater access to manpower.

In accordance with the methodology for the DOTTS bioassay, a test was considered to be valid only if egg-to-adult survival in the combined water and acetone control treatments exceeded 60%. The selection of the 60% criterion was somewhat arbitrary, but given 70 to 80% survival using similar methods in life-history experiments without toxic treatments (Blanckenhorn et al. 2008, it ensured that potential treatment effects would be assessed by comparison to controls with an acceptable number of emergent adult flies.

For the oviposition site choice experiment, a total of eight replicate groups of 12 gravid females each were assembled in 30- X 30- X 30-cm^3 insectaries (performed in Zurich, Switzerland). Each group was offered a total of eight 15- X 40- mm^2 plastic dishes for oviposition in a randomized spatial array, with each containing fresh cow dung featuring one of six concentrations of ivermectin, ranging from 0.21 to 65.7 mg/kg FW, plus one water and one acetone control. The females were given approximately 4 h to oviposit, after which all eggs laid into each dish were counted and expressed as a percentage of the total eggs laid in any one insectary.

RESULTS

Of the 13 ring tests performed (Table 1), seven exceeded the validity criterion of 60% survival in the combined water and solvent control, and three nearly met this criterion (at least 55% survival). The remaining three tests (S-6, S-10, and S-11) did not meet the criterion, although a clear dose–response pattern was observed in all of them. All data are reported here for completeness.

Ivermectin-dependent lethal effects on survival

Treatment effects were detected in each test ($p < 0.001$) (Figs. 1 and 2). The EC50s were calculated for the 10 tests that produced a pattern of reduced survival with increased ivermectin concentration (Table 1). The EC50s could not be calculated for three tests (S-6, S-7, and S-9) that did not show a dose–response effect (Table 1). The 10 calculated EC50s were within a range of 1.1 to 32.6 mg ivermectin/kg dung FW (mean +/- standard

deviation, 20.8 +/- 19.1 mg/kg FW) (Table 1). The extremely high EC50 of 67.5 mg/kg FW calculated for test S-4 is explained most easily by differences in the susceptibility of the field-collected flies used in this test versus the laboratory-reared flies from Zurich used in all other tests. The estimated NOEC survival values averaged across the same 10 tests (i.e., excluding tests S-6, S-7, and S-9) was 8.1 +/- 7.7 mg/kg FW (range, 0.25–20.2 mg/kg FW).

Ivermectin-dependent sublethal effects on development time and body size

Egg-to-adult development time increased with ivermectin concentration (all individual tests: $p < 0.001$) (Figs. 3 and 4). This pattern was observed for both males and females, although the degree of effect varied among tests (interaction test: $p < 0.001$). The NOEC for development time was one order of magnitude lower than that for survival (Table 1). Sublethal exposure to ivermectin also reduced the body mass of both male and female flies ($p < 0.001$), as documented in each of the two tests for which measurements were obtained (Fig. 5). Taken together, prolonged development and reduced body mass imply retarded larval growth in response to ivermectin residues. The results of the present study thus emphasize that sublethal effects, in addition to lethal effects, should be considered when examining the nontarget effects of parasiticide residues in dung of treated livestock.

Oviposition site choice with regard to ivermectin

Yellow dung fly females do not discriminate among oviposition sites (miniature dung pats) featuring different, sometimes lethal ivermectin concentrations (e.g., by contact toxicity) (linear regression: $p > 0.1$) (Fig. 6).

DISCUSSION

The present study documents the practicality and repeatability of the DOTTS bioassay protocol as validated for ivermectin in a ring test performed among seven laboratories in four countries. Calculated EC50s, NOECs, and development times from the European and Canadian laboratories were in general agreement. Excluding the results for test S-4, which used flies of

different genetic stock, mean values for survival differed by approximately twofold for EC50 and approximately 80-fold for NOEC in comparisons between the two sets of laboratories. These differences may have been caused by use of a formulated product and a different range of ivermectin concentrations in the Canadian tests.

Strong and James (1992) also examined the toxicity of ivermectin residue in dung to newly hatched S. stercoraria larvae. They reported EC50s of 51 and 36 mg/kg dung FW when larvae were exposed to fecal residues for 24 and 48 h, respectively. Based on the survival of these larvae to pupation and to adult emergence, EC50s were 15 and 1 mg/kg FW, respectively (Strong & James 1992). By comparison, the average estimated EC50 for adult emergence in the present study was 20.9 mg/kg FW. The lower EC50s for adult emergence reported in Strong and James (1992) may reflect differences in the sensitivities of the fly populations used, differences in the stress levels of the experimental conditions, or both.

Fecal residues of veterinary pharmaceuticals can have sublethal effects on insects breeding in dung (Floate et al. 2005). For this reason, the OECD Draft Guideline protocol (OECD 2004a) recommends measuring egg-to-adult development time as well as aspects of morphology, such as body size or wing deformations of adult flies, in addition to mortality. These measurements can be collected with only a little additional effort, and they provide a more sensitive indicator for the presence of residues. In the present study, the average estimated NOEC for adult survival was 8.1 mg/kg FW, whereas it was 0.8 mg/kg for development time, which is one order of magnitude lower (Table 1). At concentrations of 0.5 mg/kg FW, Strong and James (1992) further reported ivermectin residues to cause wing abnormalities and to increase the level of fluctuating asymmetry in wing traits, both of which, however, are more cumbersome to assess and are not as reliable (Floate & Fox 2000).

In contrast to the insensitivity of some dipteran species (e.g., nematocerans Madsen et al. 1990), the present results show that S. stercoraria is affected by levels of residue within the range expected for dung of cattle treated with recommended doses of veterinary products. The oviposition site choice further indicates, perhaps unsurprisingly given the short

time this substance has been in use (since 1981), that *S. stercoraria* cannot perceive even high, lethal ivermectin concentrations in dung, so the ovipositing females are unable to avoid any dung properties detrimental to their larvae. Cattle treated topically with ivermectin at the recommended dosage of 500 mg/kg body weight excreted residues at concentrations of 2,800 mg/kg dung FW (2 d postapplication) to 6 mg/kg FW (28 d postapplication) (Herd et al. 1996). When treated with ivermectin injections at the recommended dosage of 200 mg/kg body weight, cattle excreted residues at concentrations of 200 mg/ kg dung FW (3 d postapplication) to 10 mg/kg FW (28 d postapplication) (Herd et al. 1996). Suarez et al. (2003) investigated dung of cattle injected with doramectin or a long-acting formulation of ivermectin at a recommended dosage of 200 mg/kg body weight and found ivermectin residues of 1,150 mg/kg dung FW (3 d postapplication) to 22.8 mg/kg FW (29 d postapplication).

Because of various factors that differed naturally between the tests in the various laboratories (e.g., time of test, laboratory conditions, and handling of eggs), the specific sources of the heterogeneity in the data ultimately cannot be identified. However, where a particular factor was inadvertently or deliberately altered, something can be said. The only test using wild-caught flies as opposed to flies from the Zurich stock (test S-4) yielded substantially higher EC50 and NOEC values for mortality and development time. This could indicate greater sensitivity of the laboratory stock compared to wild flies, perhaps because of inbreeding depression or laboratory adaptation. Conversely, this result could indicate particularly strong resistance to ivermectin by the wild-caught flies, perhaps because of previous exposure to the substance. However, because this laboratory versus field effect remained unreplicated, a definitive answer cannot be provided, and it would be interesting to pursue this question further. The use of formulated (dried and rewetted) dung in test S-3, albeit again unreplicated, did not appear to produce great differences. An obvious gain in experience with handling the flies and/or with conducting the experiment can be seen from the data, especially in cases where the same laboratory performed the same test multiple times. The later tests of both the Canadian and one of the German (Rossdorf) laboratories clearly showed higher survival in the controls, and the

earlier tests generally had lower overall survival, not meeting the validity criteria specified in the guideline (Table 1).

From the results of the ring test presented here, it can be concluded that technically, the new guideline for yellow dung fly testing (OECD 2008) works very well: It is understandable, practicable, and produces replicable and comparable test results across laboratories. Because yellow dung flies can be cultured easily (Blanckenhorn et al. 2008), contrary to some dung beetles, provision of test organisms does not cause problems in principle, although no commercial supplier yet exists for this species.

There also are few problems with delivering the fly eggs, although overnight delivery incurs significant costs and eggs have to be processed immediately. Nevertheless, in response to the present ring test experiences, some improvements were identified in the older ring test version (OECD 2004a) and have been incorporated in the latest version of the guideline (OECD 2008) together with comments made by colleagues from OECD member countries during the standardization process. Finally, but importantly, an investigation of both nonlethal (development time and body size) and lethal effects is strongly recommended, because these can be evaluated easily using the same experiment with the same methods and yield valuable additional information. A similar conclusion already was agreed on in the test guidelines for sediment-inhabiting midge larvae (OECD 2004b). Similar ring tests with other test organisms, such as dung beetles (e.g., *Aphodius constans*) or other flies (e.g., *Musca autumnalis*), should follow to strengthen and generalize the results of the present study on yellow dung flies as well as the regulatory success of any guideline for dung organisms.

ACKNOWLEDGMENTS

We thank the members of the DOTTS ring test group and especially Keith Wardhaugh for commenting on drafts of the test guideline as well as Katie Barrett and Jane Grey for hosting the final evaluation workshop in Huntingdon (UK) in 2006. For financial and technical support, we gratefully acknowledge the Velux-Stiftung (Zurich, Switzerland) and Paul Cooper (Merial) who provided the test substance. Finally, the constructive role of the scientific

officers at the OECD responsible for the standardization of the dung fly test is highly appreciated.

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Table 1: Compilation of ring test results with *Scathophaga stercoraria*

Test	Valid[1] (control survival)	EC50 for survival (µg ivermectin/kg fresh dung)[5]	NOEC for survival (µg ivermectin/kg fresh dung)	NOEC for development time (µg ivermectin/kg fresh dung)	Place / Time
S-1	Yes (87.2%)	10.2 (n. d.)	4.0	0.25	Flörsheim, D/ 2004
S-2	Yes (79.2%)	14.3 (10.9 – 18.9)	6.4	0.64	Flörsheim, D/ 2004
S-3[2]	Yes (72.9%)	25.7 (18.1 – 36.6)	16.0	1.00	Flörsheim, D/ 2005
S-4[3]	Yes (94.2%)	67.5 (51.2 – 89.0)	20.2	2.00	Yorkshire, UK/ 2006
S-5	Yes (70.3%)	21.5 (16.9 – 27.5)	6.4	0.64	Niefern, D/ 2005
S-6	No (42.0%)[4]	n. d.	n. d.	1.11	Rossdorf, D/ 2004
S-7	Nearly (55.0%)[4]	n. d.	n. d.	1.00	Rossdorf, D/ 2006
S-8	Yes (74.4%)	32.6 (23.4 – 45.6)	20.2	0.64	Zürich, CH/ 2006
S-9	Nearly (57.5%)[4]	n. d.	n. d.	6.40	Itingen, CH/ 2006
S-10	No (34.2%)	22.4 (0.00007 – 109.4)	2.5	2.5	Lethbridge, CAN/ 2003
S-11	No (22.1%)	1.05 (0.67 – 1.65)	0.25	0.25	Lethbridge, CAN/ 2004
S-12	Nearly (57.4%)	7.24 (n. d.)	2.5	0.25	Lethbridge, CAN/ 2005
S-13	Yes (63.7%)	6.33 (n. d.)	2.5	0.25	Lethbridge, CAN/ 2005
Mean		20.9 ± 19.1 (N = 10)	8.1 ± 7.7 (N = 10)	0.84 ± 0.79 (N = 10)	

1) Validity criterion = 60 % survival according to guideline; 2) formulated dung; 3) wild-caught flies; 4) included since the effect followed a dose-response-pattern; 5) standard deviation is given in parentheses; EC50 = median effective concentration; n.d. = not determinable; NOEC = no-observable-effect concentration.

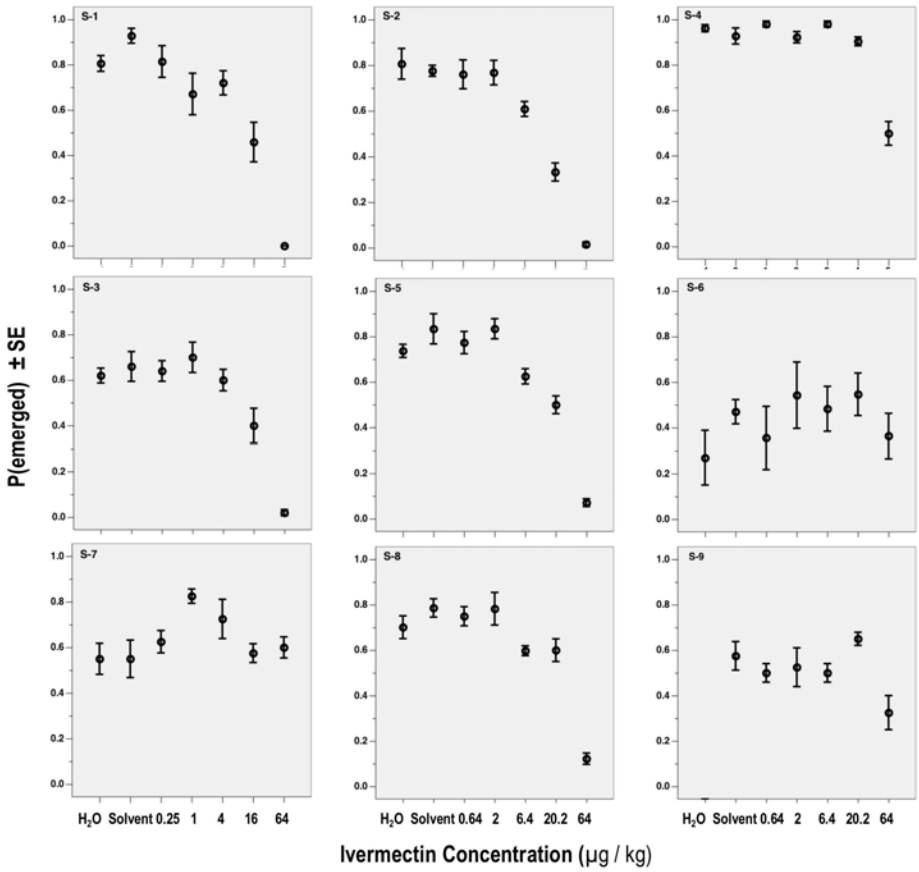

Fig. 1: Proportion (mean ± standard error [SE]) of yellow dung flies (*Scathophaga stercoraria*) emerged (larva-to-adult survival) as a function of ivermectin concentration (plus two controls) for the nine European ring tests (S-1 to S-9). Note the slightly different concentrations in the three leftmost panels. Also note that test S-9 had no water control.

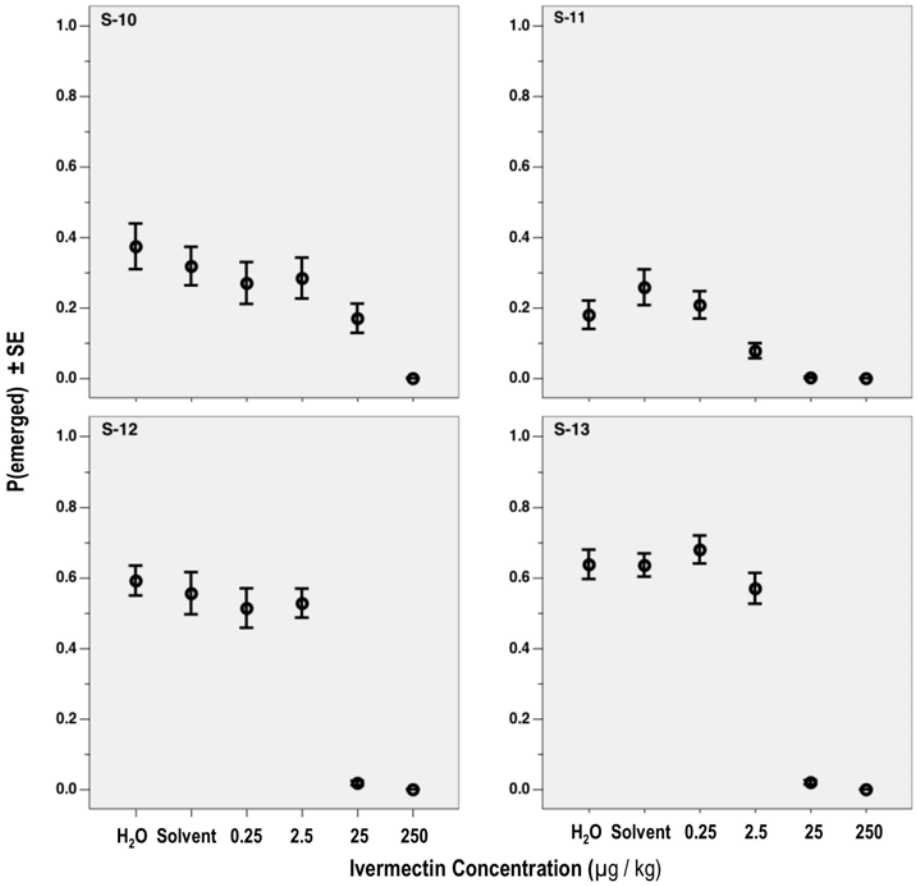

Fig. 2: Proportion (mean ± standard error [SE]) of yellow dung flies (*Scathophaga stercoraria*) emerged (larva-to-adult survival) as a function of ivermectin concentration (plus two controls) for the four Canadian ring tests (S-10 to S-13).

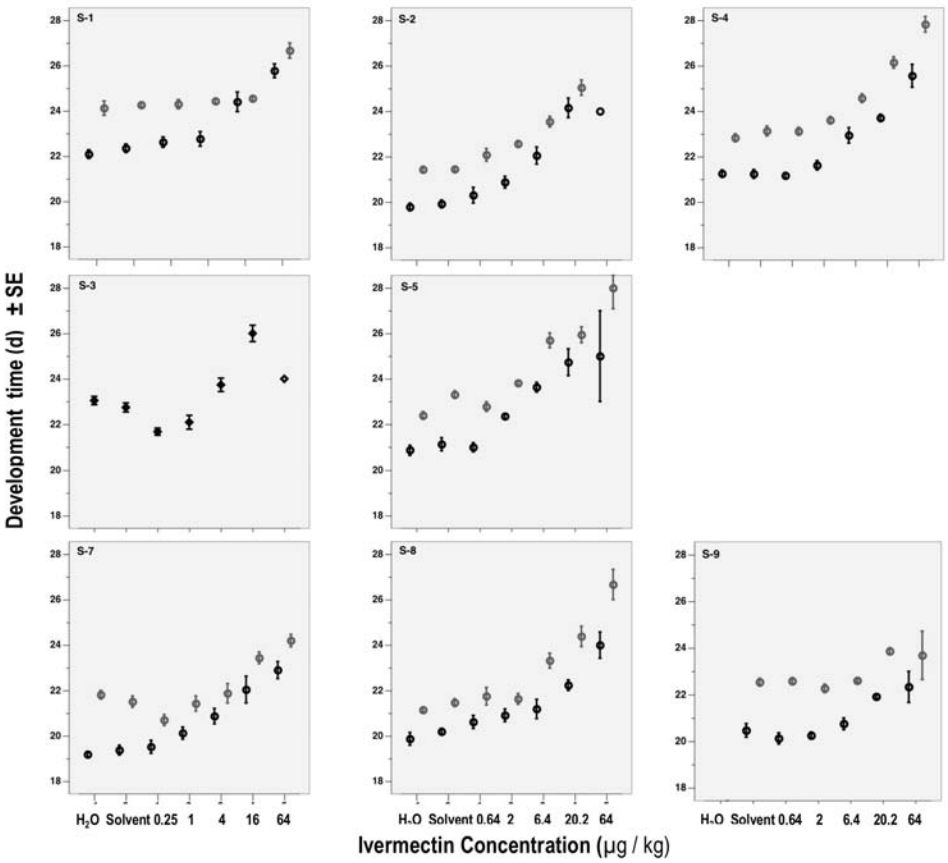

Fig. 3: Egg-to-adult development time (d; mean ± standard error [SE]) of yellow dung fly (*Scathophaga stercoraria*) females (black circles) and males (grey circles) as a function of ivermectin concentration (plus two controls) for eight European ring tests (S-1 to S-9). Note that test S-3 did not differentiate between the sexes. Also note that test S-9 had no water control.

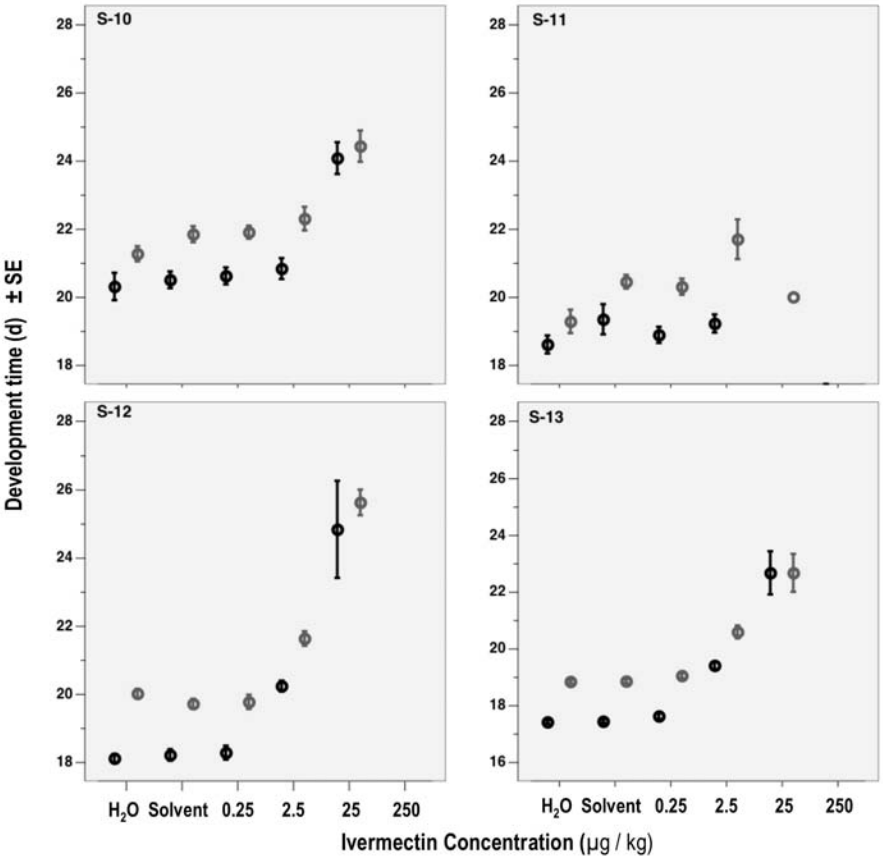

Fig. 4: Egg-to-adult development time (d; mean ± standard error [SE]) of yellow dung fly (*Scathophaga stercoraria*) females (black circles) and males (grey circles) as a function of ivermectin concentration (plus two controls) for the four Canadian ring tests (S-10 to S-13).

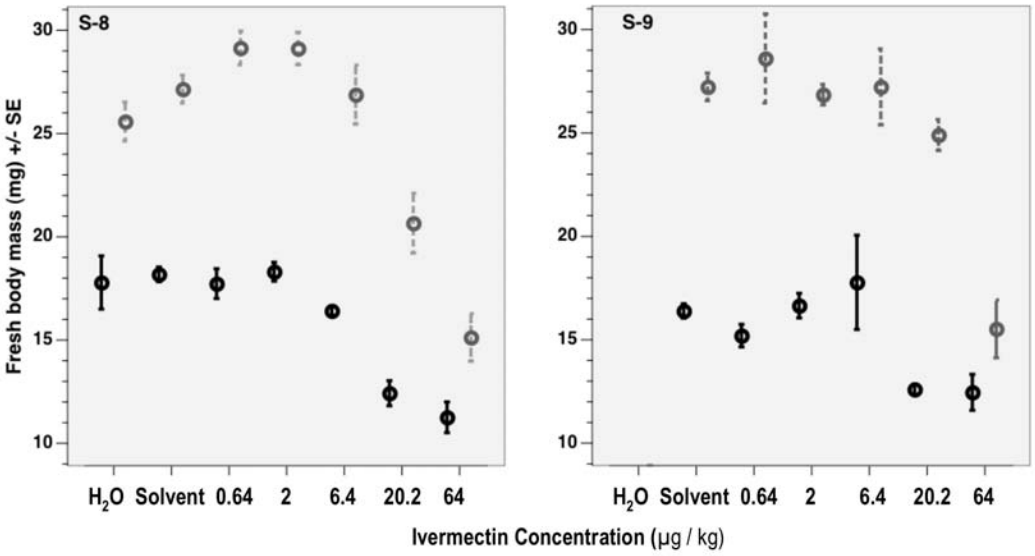

Fig. 5: Fresh teneral adult body mass (mg; mean ± standard error [SE]) of yellow dung fly (*Scathophaga stercoraria*) females (black circles) and males (grey circles) as a function of ivermectin concentration (plus two controls) for two European ring tests (S-8 to S-9). Note that test S-9 had no water control.

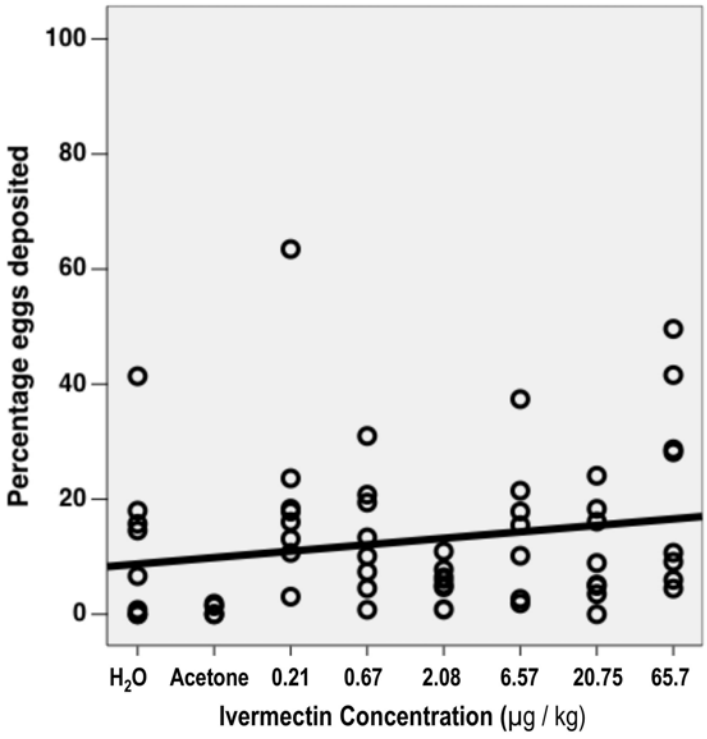

Fig. 6: Percentage of eggs laid by yellow dung flies (*Scathophaga stercoraria*) into one of eight dung dishes containing dung of various ivermectin concentrations plus two controls (water and acetone) by eight replicate groups of 12 gravid females each.

Assessing the effect of pharmaceutical residues on biodiversity at the landscape level

R. Jochmann and W. U. Blanckenhorn

ABSTRACT

Many pharmaceutical residues are continuously released into the environment. They can have strong detrimental effects on natural ecosystems, especially if they were designed to fight pest organisms. Therefore, testing the environmental effects of such residues is an important task. To do this, different strategies exist. The most common is to test single species in the laboratory. Yet, as the residues will affect whole ecosystems, a more realistic approach is to also directly assess the effect on these ecosystems. Every species is embedded in a network of biotic and abiotic interactions that will, in most cases, not be represented in single species bioassays in the laboratory. We conducted a large-scale field experiment at the landscape scale (25 cattle pastures north of the Swiss Alps) to test the effect of the anthelminthic ivermectin on the cattle dung insect community over the season (spring, summer, autumn). Comparing replicated paired ivermectin-spiked (6.6µg ivermectin / kg wet dung) vs. control dung pats, ivermectin overall strongly reduced the number of taxa emerging from the dung as well as the Shannon diversity index, while the total number of individuals emerged did not change. This reduction was not apparent in autumn, when fewer taxa were present, and also not in some of the pastures. Various characteristics of the pasture (elevation, vegetation cover, humidity, soil fertility) could not explain these effects. We conclude that, at the landscape level in the natural environment, ivermectin kills a number of sensitive dung taxa, thus reducing dung biodiversity and ultimately the decomposition of dung. Our study shows that it is not only important to assess environmental effects of toxic substances on whole ecosystems, but also to replicate such studies in space and time.

INTRODUCTION

Biodiversity is endangered by an array of environmental threats, many of which relate to human activities. A prevailing common threat is the release of synthetic chemical compounds into the environment. Many of these compounds are released intentionally, like pesticides to fight insects feeding on field crops or artificial fertilizers to increase crop yields. To these add substances that reach the environment unintentionally, like antibiotics used to fight diseases. The side effects on natural ecosystems can be severe (Ratcliffe 1970, Kidd et al. 2007) and possibly outweigh the benefits, e.g. a reduction in crop-yield when originally intending to increase it (Fox et al. 2007).

A particularly common class of environmental pollutants that likely disturb ecosystems are pharmaceutical residues. Livestock animals are treated regularly with a large selection of different pharmaceuticals that often end up in the environment. Such medications particularly impact on ecological communities directly associated with livestock breeding, such as dung breeding organisms, but also on communities that are not directly related to farming (Boxall et al. 2005). Many pharmaceuticals are excreted with the dung, and therefore the dung ecosystem has been subjected to pharmaceutical residues for many decades. A frequent approach to estimate the impact of a pharmaceutical has been to test it on single species in laboratory bioassays (e.g. Gover & Strong 1996, Wardhaugh et al. 1996, Krüger & Scholtz 1997, Wardhaugh & Mahon 1998, Wardhaugh et al. 2001a, Wardhaugh et al. 2001b, Lumaret et al. 2007, Kolar et al. 2008). A severe shortcoming of this method is the subjective selection of the species to be tested. Often well-established laboratory species are used for these tests (e.g. Römbke et al. 2009). Yet, sensitive taxa are generally more challenging to breed in the laboratory, which leads to a bias in the representation of sensitive and non-sensitive species in standard toxicological studies. Furthermore, all members of a community are linked to each other in a complex network of competition and predation (Pimm 1980), and typically some species are more affected by a given pharmaceutical than others. This will ultimately lead to changes in the ecosystem not predictable from laboratory experiments. Therefore, it is indispensable to assess the effect of any pharmaceutical on

the whole ecosystem in the field. Moreover, the dung ecosystem, like any other ecosystem, differs in species composition between regions, seasons and years (e.g. Finn et al. 1998, Sánchez Piñero & Avila 2004). Factors like vegetation and altitude can cause alterations in species composition (Menéndez & Gutiérrez 1996). This means that results obtained for one locality or for one season cannot necessarily be generalized. Preferably, a field study should include all the seasons, more than one year and as many different localities as possible, with different climate, altitude, vegetation cover, soil fertility etc.

One of the most common pharmaceuticals employed in livestock breeding is ivermectin, a parasiticide applied against nematodes and arthropods. Ivermectin has been used as a case substance in various eco-toxicological tests in the past, ranging from single-species laboratory tests (e.g. Römbke et al. 2009) to single location field tests of entire ecological communities (e.g. Suárez, 2003; Kryger, 2005). Ivermectin is excreted with the dung of the treated animal (Alvinerie et al. 1998), maintaining its toxicity (Wall and Strong 1987). While most arthropod taxa tested in laboratory assays are negatively affected (e.g. Cook 1991, Errouissi et al. 2001, Gover & Strong 1995, Miller et al. 1981; Römbke et al. 2009, 2010a), in field studies some taxa seem to be indifferent towards the substance (Floate 1998a, Römbke et al. 2010b, Schmidt 1983, Sommer 1992, Strong et al. 1996), or may even show increased survival (Iwasa et al. 2005), potentially due to release from inter-specific competition or predation.

Here we present the results of a systematic landscape study of the effect of ivermectin on the diversity of the cattle dung insect community, including 25 different pastures, three different seasons (spring, summer, autumn) over two different years. The concentration we used was relatively low, corresponding, for example, to one that can be found in the dung of treated cattle approximately one month after pour-on treatment with 0.5mg ivermectin per kg body weight (Fernandez et al. 2009, Herd et al. 1996). We show that the effect on the insect community still is very powerful, with diversity measures being strongly reduced. This raises the question of whether it would be possible to balance the negative effects of parasites and

the negative environmental side effects by reducing the amount of ivermectin applied. Moreover, our study shows that firm conclusions on the environmental side effects of pharmaceutical residues (and all other residues released into the environment) must be based on large-scale landscape studies that cover different seasons, localities and ecosystems.

MATERIAL AND METHODS

We had two treatments, an untreated control cattle dung pat and an ivermectin-treated pat, repeated over three seasons (spring, summer, autumn). Each treatment-by-season combination was replicated twice within each of 25 cattle pastures. The study was conducted over two years in 2007 and 2008. The pastures were distributed across northern Switzerland (Fig 1) and subjected to different farming practices. Their elevation ranged from 360-1130m.

Dung treatment

We collected and homogenized fresh dung from the cattle present on the pasture (which were untreated), and mixed 1mL of acetone per 600g of dung. In the ivermectin treatment, the acetone contained dissolved ivermectin to obtain a concentration of 6.6µg ivermectin per kg of dung (wet weight). The control treatment had pure acetone. Dung was partitioned into 600g pats and placed on five liters of soil contained in a plastic bowl. The bottom of the bowl was perforated to allow water to drain while preventing organisms from escaping. The two replicate pairs of pats were situated at opposite ends of the pasture. There was about 5m distance between the control and ivermectin pats within a pair, ensuring that they were located in the same microhabitat and subjected to the same microclimate. Dung pats were left in the field for one week, enabling colonization and egg-deposition. Each pat was then transferred, together with the soil contained below, into an emergence box. The purpose of these boxes was to capture all organisms completing their development in the dung from egg to adult during the following four weeks. Therefore, the boxes had only one exit, leading all emerging insects into a container filled with 70% ethanol. For this study, we focused on species of

Diptera and Hymenoptera, as Coleoptera have already been extensively investigated.

Pitfall traps

The dung pats described above (henceforth called emergence traps) were each accompanied by a dung-baited pitfall trap nearby, the bait dung treated like that of the associated dung pat. These traps, in contrast to the emergence traps, captured adult organisms attracted to the dung. This served to assess if any taxon was attracted or repelled by ivermectin-spiked dung, which would distort results of the emergence traps (see Floate 1998b, 2007, Holter et al. 1993, Wardhaugh & Mahon 1991). The taxonomic composition of the two trap types was not expected to be identical, as pitfall traps would capture accidental and cursory visitors of the dung.

Statistics

Per pat we computed three measures describing biodiversity of a community, number of taxa (taxa richness), number of individuals (abundance), and the Shannon index of taxon diversity, which combines information about taxa numbers and abundance (Pielou 1974). Note that the taxonomic levels of the groups differ, some being species, others subsuming genera or families; however, this should not introduce systematic bias. The three diversity measures were analyzed with ivermectin treatment and season as fixed (repeated) factors, pasture (the unit of replication) as a random effect, and various characteristics of the pasture (elevation, vegetation cover, humidity, soil fertility) as potential covariates.

RESULTS

Pitfall traps

When comparing the number of individuals attracted to untreated and ivermectin-treated cattle dung, we found that none of the taxa also occurring in the emergence traps was attracted or repelled by ivermectin-treated dung (Fig 2).

Emergence traps

Ivermectin had a negative impact on many but not all taxa, although no taxon benefited from ivermectin presence. Out of 41 taxa, the emergence of 12, or 30%, was significantly reduced in ivermectin-treated dung (Fig 5).

Ivermectin strongly reduced the number of emerged taxa as well as the Shannon diversity index subsuming number of taxa and their abundance, while the number of individuals emerged did not change (ivermectin effect in Table 1; Fig. 3). For the two former measures, there was an interaction of ivermectin with the season in that the reduction was less pronounced in autumn than in spring or summer (season by ivermectin interactions in Table 1; Fig. 3). The number of taxa emerged was also lower in autumn, which was only marginally true for the number of individuals or the Shannon index (season effect in Table 1; Fig. 3). Lastly, all three indices varied among years and pastures (latter effect marginal for the Shannon-index; Table 1). Nevertheless, the effect of ivermectin on the Shannon-index varied for the 25 pastures (ivermectin by pasture interaction being marginally significant), with no difference visible on some pastures (Fig 4). No significant effects of any covariate were found, so they were all dropped from the final model.

To identify the reasons for the reduced effect of ivermectin in autumn (Fig 3), we checked whether the proportion of significantly reduced taxa differed between the seasons. We found no difference in this proportion, although the significantly reduced taxa showed a trend to be less abundant in autumn, but also in spring.

DISCUSSION

Our study clearly shows that the effect on biodiversity of even a low concentration of ivermectin in the dung is strong. Notably, the concentration employed in this study occurs in the dung long (> 4 weeks) after application of ivermectin to cattle. Residue concentrations can easily be 10 to 100 times higher under conventional treatment regimes before this point in time (e.g. Herd et al. 1996, Alvinerie et al. 1998), implying that studies like ours testing lower concentrations probably underestimate the effect of ivermectin on dung biodiversity. Furthermore, our study demonstrates that the effect of ivermectin residues in dung can vary between localities and seasonally. We

hypothesized that the variation among localities might be explained by various covariates such as elevation, vegetation cover, humidity, or soil fertility. Yet no significant correlation between any of the covariates and any biodiversity measure was found, although the power of our test was naturally limited with n = 25 farms. Thus the reasons for differences in the effect of ivermectin among the pastures remain unexplained.

Importantly, our data show that attraction to dung was not affected by ivermectin (Fig 2). We therefore conclude that reductions in the emergence data are not due to behavioural avoidance, but rather to mortality caused by direct or indirect effects of ivermectin.

The fact that the number of individuals emerging did not change due to ivermectin treatment (Fig 3) suggests that one or more taxa replace the ivermectin-sensitive taxa in treated dung. The fungus gnats (Sciaridae) had approximately 5500 individuals more in ivermectin-treated dung compared to control dung (Fig 6), with the fraction of fungus gnats increasing from 60% to 68.4%, although this increase was not significant (Fig 6). Nevertheless, the increased total number in this taxon might be responsible for the constant number of individuals in total in both treatments, a shift that might be explained by reduced competition.

Ivermectin reduces the biodiversity of the cattle dung insect community in terms of taxa richness and the Shannon diversity index, but the reduction in autumn is slight. In general, the number of taxa in autumn is already greatly reduced also in the untreated dung: only 25 taxa emerged in autumn, compared to 42 taxa in spring and 39 taxa in summer, with all taxa present in autumn also present in spring and summer. The reduced sensitivity could be explained if among the remaining taxa in autumn there were disproportionately more non-sensitive taxa. In other words, if fewer sensitive taxa (as defined by those showing a significant reduction; cf. Fig. 2) emerged in autumn, e.g. because they hibernated more often as eggs, larvae or pupae, the effect of ivermectin would be reduced. However, our data do not support this hypothesis because the proportion of sensitive taxa did not differ significantly between the seasons. The reduced effect of ivermectin in autumn must therefore have another reason; perhaps it is a mere statistical consequence of the lower number of taxa present in autumn. Similarly, while

on most pastures ivermectin reduced the Shannon-index, on some pastures this effect was not present. These results clearly show that assessment in a single season or at a single locality can be misleading and that studies should include several sites and seasons to be representative.

In summary, our study demonstrates at the large, landscape scale that, despite some expected spatio-temporal variation, livestock medications inadvertently excreted into the environment do profoundly disturb the composition of this community, which, although the precise mechanisms are unclear, will impact on its ecosystem function, most notably the decomposition of dung. We suggest that more such field studies replicated in space and time be conducted with other substances and in other areas to generalize our results. We further suggest evaluating whether reduced application of ivermectin, though probably resulting in less effective parasite control, could balance the negative target effects on parasites and the negative non-target environmental side-effects on the dung community.

ACKNOWLEDGMENTS

We thank the Velux foundation for generously financing this PhD project. We also thank Thomas Walter, Agroscope Reckenholz-Tänikon (CH), for supplying access to and data on the pastures studied during this project.

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Table 1: Analysis of variance results for the three used measures describing dung biodiversity.

Number of individuals (square root transformed)				
factor	**df**	**MS**	**F**	**p**
year	1	53,863	62383	<0,001
pasture (year)	23	0,867	4,309	<0,001
ivermectin	1	0,611	3,02	0,095
season	2	0,813	2,524	0,082
season * ivermectin	2	0,051	0,158	0,854
pasture * ivermectin	24	0,201	0,625	0,914
error (global)	229	0,322		
Number of taxa				
factor	**df**	**MS**	**F**	**p**
year	1	147,407	6,51	0,018
pasture (year)	23	22,732	3,756	0,001
ivermectin	1	355,549	58,518	<0,001
season	2	276,498	31,377	<0,001
season * ivermectin	2	47,684	5,411	0,005
pasture * ivermectin	24	6,051	0,687	0,862
error (global)	229	8,812		
Shannon-index				
factor	**df**	**MS**	**F**	**p**
year	1	8,772	22,19	<0,001
pasture (year)	23	0,397	1,852	0,070
ivermectin	1	5,905	27,623	<0,001
season	2	0,346	2,368	0,096
season * ivermectin	2	0,706	4,835	0,009
pasture * ivermectin	24	0,214	1,469	0,079
error (global)	229	0,146		

Fig. 1: Map of Switzerland with the location of the 25 pastures.

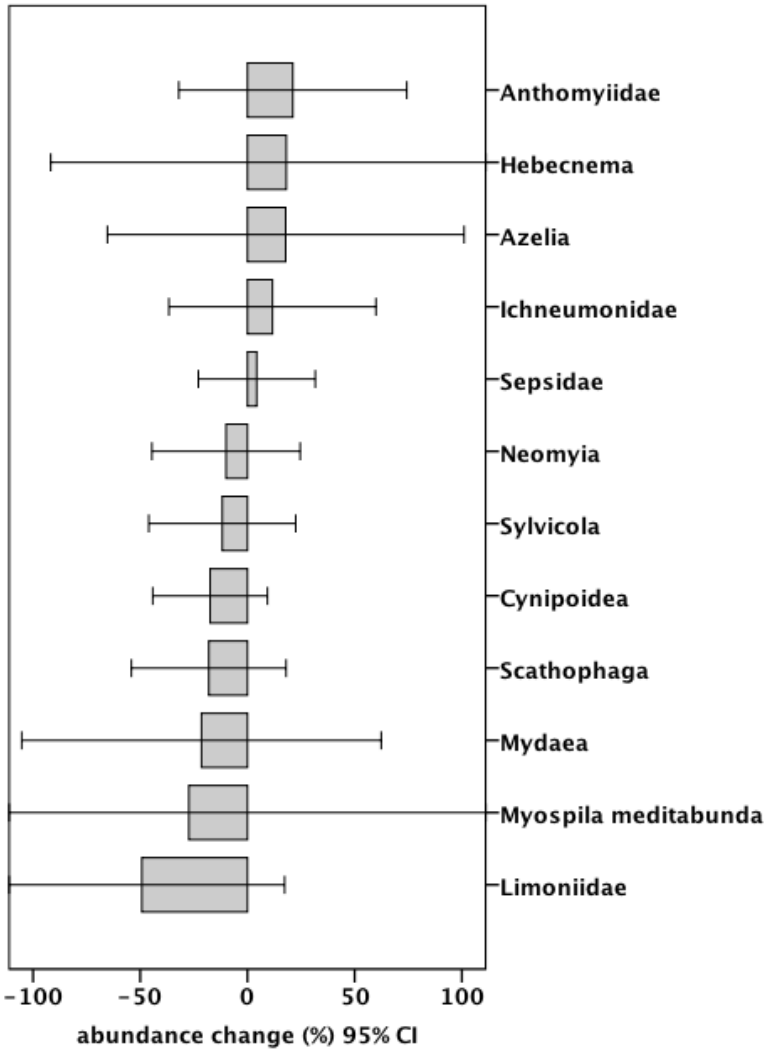

Fig. 2: Abundance changes (percent) of taxa attracted to cattle dung after treatment of the dung with ivermectin. The number of individuals for each taxon captured with control dung was subtracted from the number of individuals captured with ivermectin-treated dung and divided by their sum. Taxa repelled by ivermectin are below zero; taxa attracted are above zero. Only the taxa also present in the emergence traps are plotted (cf. Fig. 5)

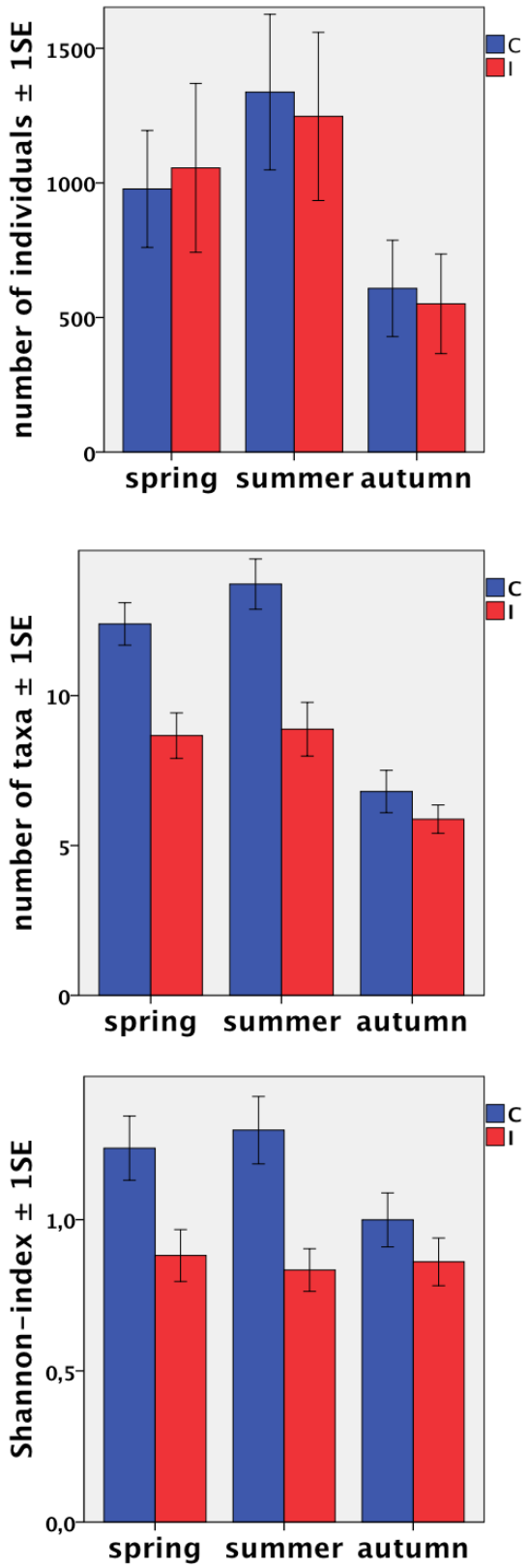

Fig. 3: Variation in number of individuals, number of taxa and Shannon index, as calculated from the taxa emerging from cattle dung, in relation to season and ivermectin treatment (C = control dung, I = ivermectin-treated dung).

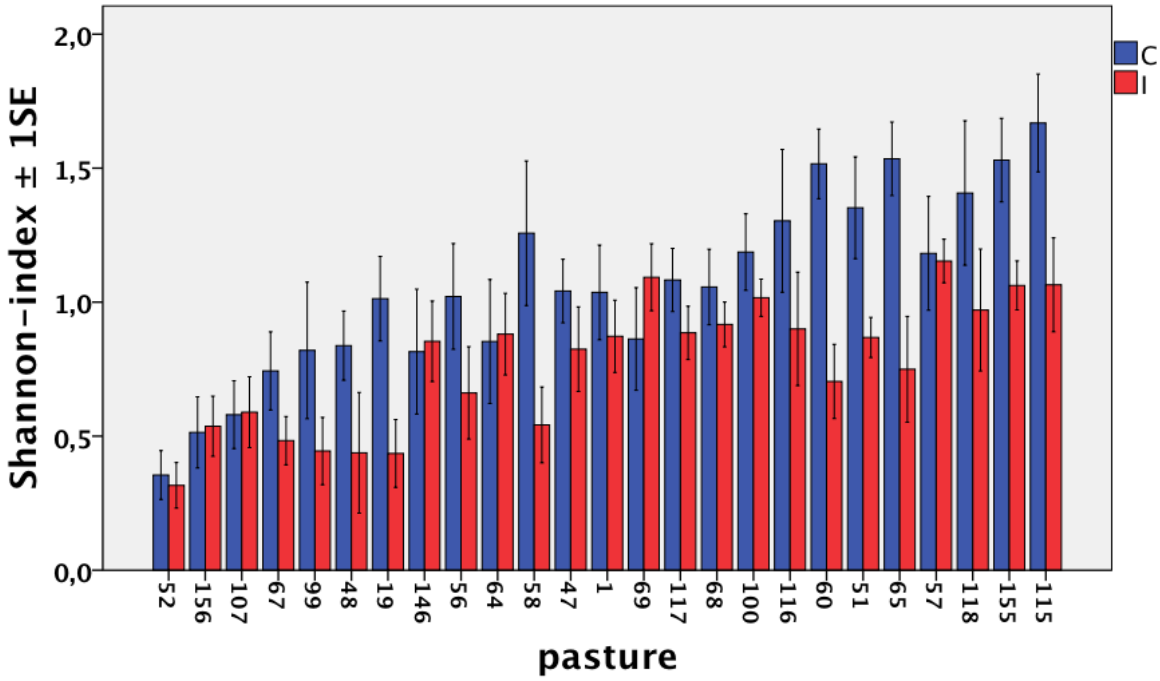

Fig. 4: Variation in the Shannon index, as calculated from the taxa emerging from cattle dung, for the 25 pastures and treatments (C = control dung, I = ivermectin-treated dung).

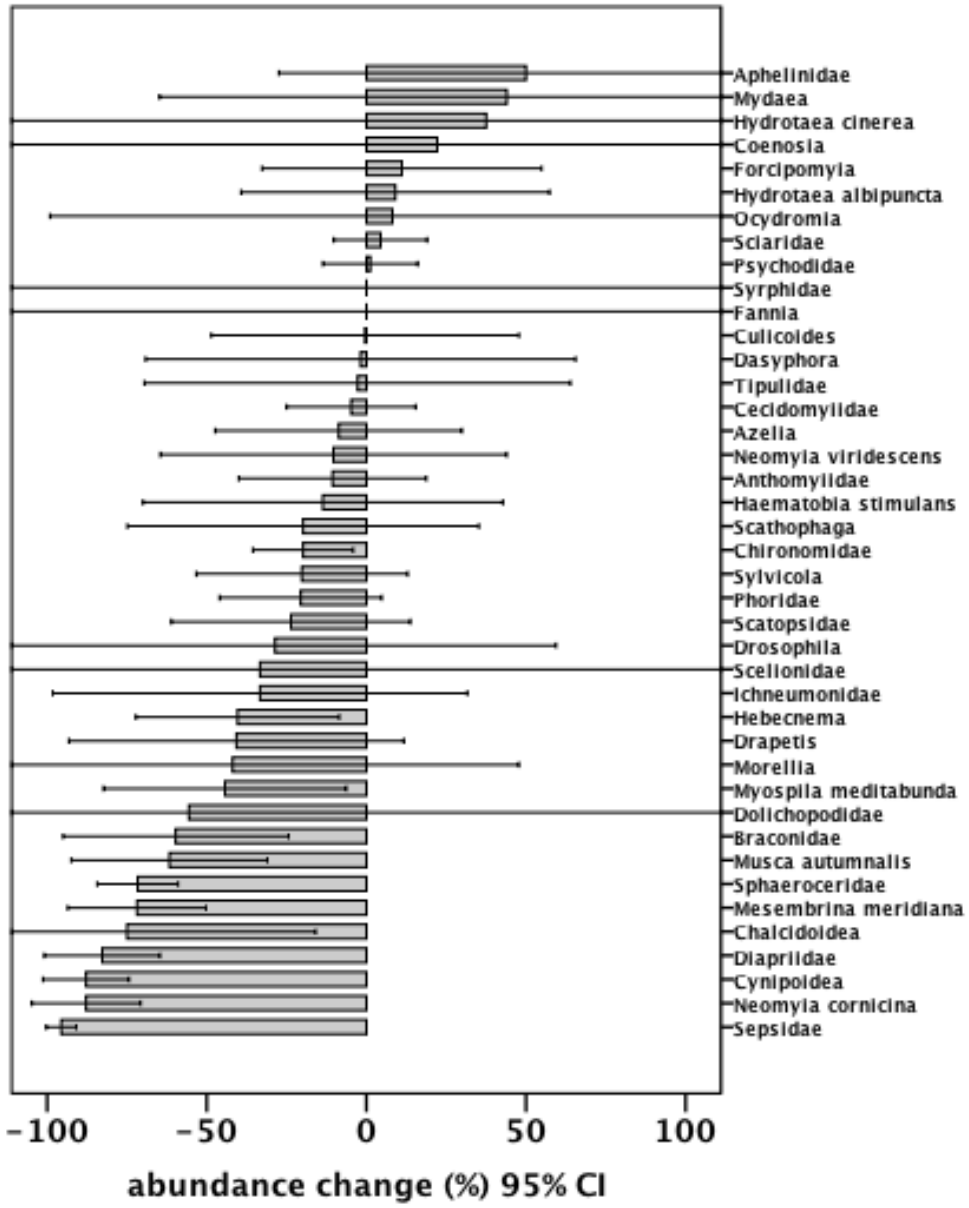

Fig. 5: Abundance changes (percent) of taxa emerging from cattle dung after treatment of the dung with ivermectin.

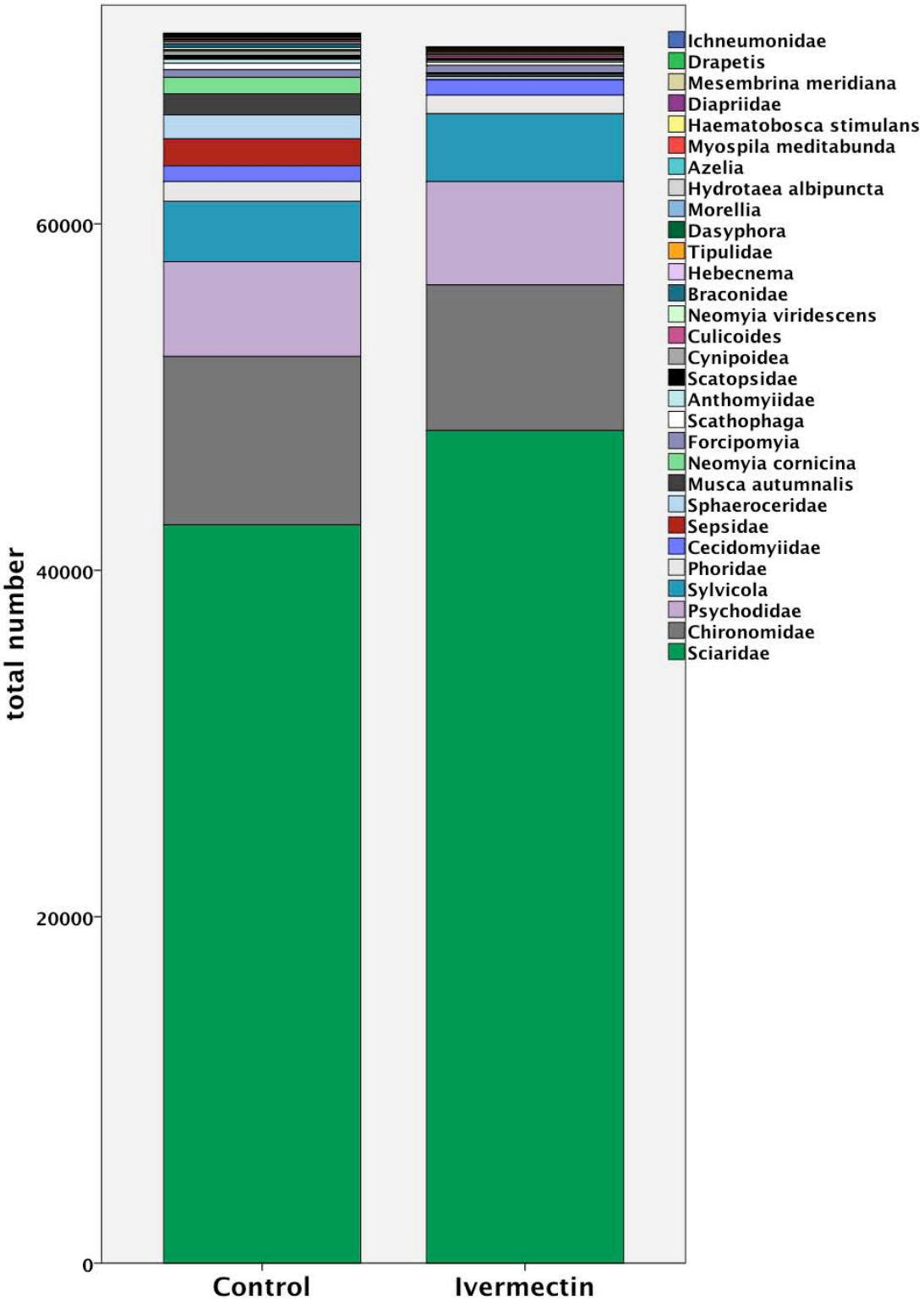

Fig. 6: Total number of individuals of the taxa that emerged from cattle dung in relation to ivermectin treatment.

Ivermectin unequally affects trophic groups of the dung community

R. Jochmann and W. U. Blanckenhorn

ABSTRACT

Natural or man-made environmental stressors often have negative effects on the fitness of organisms. Selective killing or differential sensitivity of particular species can ultimately disturb the whole complex ecosystem. Theory predicts that organisms at top trophic levels of a community (notably predators) are particularly sensitive to environmental disturbance, simply because their numbers tend to be low and their body size large, but also because pollutants can biomagnify in the food chain. We investigated the effect of ivermectin, a common anti-parasiticide, on the cattle dung insect community in a large-scale field experiment at the landscape level (25 pastures). The cattle dung pat harbours a discrete ecological community of a limited number of interacting organisms (primarily insects) of various trophic groups that is amenable to experimentation. We found a graded negative impact of ivermectin on many but not all groups, although no taxon benefited from ivermectin presence. Hymenopteran parasitoids decreased particularly in numbers when ivermectin was present, but dipteran predators were not more reduced than coprophagous and saprophagous taxa. Thus, the investigated community only partly confirms theoretical expectations, and the effect of low population numbers seems to be less important. Hymenopteran sensitivity could be due to phylogeny rather than trophic position.

INTRODUCTION

Species show different sensitivities to disturbance, ultimately resulting in changes in their abundance and subsequently in the composition of communities and whole ecosystems (Paine 1966). This can have severe consequences for ecosystem functioning, for example through alterations in the food web (Odum 1985, Pimm 1982). In several comprehensive studies, top trophic levels were more sensitive than lower trophic levels to various forms of disturbance such as habitat fragmentation (Gonzalez & Chaneton 2002), habitat modification (Wright & Coleman 1993), or temperature increase

(Petchey *et al.* 1999). One of the most prevailing and important form of man-made pollution occurs through chemicals. For the effect of chemical residues, existing studies are restricted to the top trophic level or even single species (e.g. Ratcliffe 1963). To fill this gap, we conducted a systematic field study of the effects of ivermectin, one of the most prominent anthelminthic livestock drugs, on the various trophic levels of the cattle dung community.

Ivermectin is widely used worldwide for the control of intestinal nematodes and arthropod ectoparasites in livestock. It is excreted with the dung of the treated animal (Alvinerie *et al.* 1998), maintaining its toxicity (Wall & Strong 1987). The cattle dung community is a cryptic but compact and well-known ecosystem (e.g. Skidmore 1991), and therefore experimentally tractable to test for general effects of especially man-made disturbances on community composition and function. Over the season we repeatedly introduced cattle dung with and without ivermectin on 25 pastures in Switzerland and assessed the diversity of various arthropod groups. We aimed to evaluate the relative impact of ivermectin on the various trophic levels. We predicted that predators and parasitoids would be disproportionately reduced by the livestock medication residues, as has been shown for other ecosystems in response to disturbance (e.g. Petchey *et al.* 1999). This is expected to occur because top-level predators tend to be large and their populations relatively small (Cohen *et al.* 2003), and/or because pollutants tend to biomagnify up the food chain (e.g. Connel 1990). Unexpectedly, we found that parasitoids, but not predators, were particularly sensitive, and we discuss alternative hypotheses to explain the observed patterns.

MATERIAL AND METHODS

We studied 25 cattle pastures subject to various farming practices distributed across northern Switzerland, encompassing an area of approximately 200 x 100 km and ranging from 370-1130 m elevation (see Fig. 1, chapter 3). Our study was conducted over 3 years, with 12 pastures investigated in 2007, 12 in 2008, and one additional pasture in 2009.

The experimental design consisted of a factorial design with two paired treatments repeated three times over the season (spring, summer, autumn).

The treatments were an untreated control dung pat and an ivermectin-treated pat; each treatment-season combination was replicated twice within each of the 25 pastures. We collected and homogenized fresh dung from the cattle present on the pasture, and mixed in 1 mL of acetone per 600 g dung. In the ivermectin treatment, the acetone contained dissolved ivermectin to obtain a concentration of 6.57µg ivermectin per kg dung (wet weight), corresponding to a concentration measured approximately one month after pour-on treatment with 0.5 mg ivermectin per kg body weight (Fernandez et al. 2009, Herd *et al.* 1996). The control dung was treated with pure acetone. 600g of dung were placed on 5 L of soil contained in a plastic bowl. The bottom of the bowl was perforated to allow water to drain while preventing organisms from escaping. On each pasture we put out two replicate pairs of pats, situated at opposite ends of the pasture. There was about 5 m distance between the control and ivermectin treatments within a pair, ensuring that they were located in the same microhabitat and subjected to the same microclimate. Dung pats were left in the field for one week, enabling colonization and egg deposition. Each pat was then transferred together with the soil below into an emergence box. These boxes had only one exit, leading all emerging insects into a container filled with 70% ethanol, thus capturing all organisms completing their development from egg to adult in the dung during the following four weeks. The emergence boxes were stored at temperatures characteristic for the season. This procedure excludes any insects with juvenile periods longer than four weeks and with obligate diapause (i.e. many beetles).

The open dung pats were each accompanied by a dung-baited pitfall trap nearby, the bait dung treated like that of the associated open dung pat. These traps, in contrast to the open dung pats, captured adult organisms attracted to the dung. This served to assess if any species was more or less attracted or repelled by ivermectin-spiked dung, which could bias the emergence results of the open dung pats (e.g. Floate 2007). The pitfall traps caught beetles but probably fewer flies than the open dung pats, so the taxonomic composition of the two trap types was not expected to be identical. We restricted our analysis to Diptera and Hymenoptera, because several Coleoptera were likely excluded from the emergence traps.

The unit of analysis was the mean number (of two replicates) of

individuals per taxon, summed over the whole season for each pasture to cover differences in phenology. Every taxon was further assigned to a trophic (feeding) group (Table 1): predators, parasitoids (combined making up the top trophic level), and various lower trophic levels (coprophagous, mycophagous, saprophagous species). Predatory and parasitoid taxa in the dung community typically feed on other insect larvae or pupae (Skidmore 1991). Information on the actual diet of species of the lower trophic levels is scarce, but most species probably feed on bacteria, protists, fungi or nematodes, rather than undigested plant fibers in the dung. Therefore, for example, the taxon *Culicoides*, known to be bacterivorous and nematophagous, is included in the lower trophic level in our analysis. Our sampling thus yielded one mean difference (ivermectin vs. control) value per pasture (N = 25) and taxon nested in trophic group in our final analysis, equivalent to analyzing a paired or repeated-measures design. Note that the taxonomic levels of the groups differ, some being species, others subsuming genera or even families; however, this should not introduce systematic bias (Table 1).

We estimated the population sizes of all taxa using the numbers of adults emerged from the dung. We only included the ivermectin-free, i.e. undisturbed dung pats for this purpose, summing the numbers of individuals for each taxon per pasture to generate a mean value over all 25 pastures.

RESULTS

In the dung-baited pitfall traps, set up to detect attraction or repellence effects of ivermectin, twelve taxa also emerged from the dung pats. None of these twelve taxa showed a significant difference in attraction to ivermectin-treated vs. control dung (see Fig. 2, chapter 3).

From the open dung pats, 46 taxa of Diptera and Hymenoptera emerged altogether. 23 of these 46 taxa belong to the lower trophic levels, 13 are predators and 10 parasitoids (Table 1). For the following analyses, the taxa within trophic levels averaged over all pastures; rather than the pastures as in Fig. 1, chapter 3) are the statistical replicates. This is because taxa would be weighted by their abundance when ignoring taxonomic grouping, thus biasing the results towards common taxa, whereas in our analyses all taxa were weighted equally. Furthermore, not all taxa occurred at all pastures.

A total of 16 taxa (3 of the lower trophic level, 7 predators, and 6 parasitoids) occurred in fewer than 10 (of 150) pairwise samples (control-ivermectin), with very few individuals (mostly one) per trap. These were excluded so as to not bias the results in favor of rare taxa, leaving 30 taxa (20 lower trophic level, 6 predators, and 4 parasitoids) for the final analyses.

As indicated by non-overlap of 95% confidence intervals with zero in Fig. 1a, emergence of all trophic groups on average was significantly reduced by ivermectin. One-way analysis of variance indicates significant variation in the effect of ivermectin on the three trophic groups (Fig. 1a; $F_{2,27} = 3.30$, $p = 0.050$; the equivalent non-parametric Friedmann test yielded $Chi^2 = 6.61$, $p = 0.037$). Post-hoc comparisons show that parasitoid taxa (N = 4) were reduced more strongly than the lower trophic level (N = 20) taxa ($t = 2.87$, $p = 0.034$; non-parametric Mann-Whitney U-test $Z = 2.32$, $p = 0.018$), while this was not the case when comparing predatory (N = 6) and lower level taxa ($t = 1.50$, $p = 0.177$; Mann-Whitney-U-test $Z = 1.28$, $p = 0.257$; Fig. 1a). Note that when combining the two higher level predator and parasitoid trophic groups (N = 10 taxa) to test the original hypothesis, ivermectin more strongly reduced the higher than the lower trophic level ($t = 2.25$, $p = 0.037$; Mann-Whitney U-test $Z = 2.33$, $p = 0.019$).

Using the average numbers of individuals emerging from the untreated dung pats, population sizes for the top trophic level taxa (predators and parasitoids combined) were significantly lower than those of the lower trophic level ($t = 2.67$, $p = 0.014$; Mann-Whitney-U-test $Z = 2.02$, $p = 0.044$; Fig. 1b). One-way analysis of variance on the three trophic groups also reveals significant variation (Fig. 1b; $F_{2,27} = 4.31$, $p = 0.020$; Friedmann test: $Chi^2 = 12.98$, $p = 0.002$). Post-hoc comparisons show that both predatory ($t = 2.87$, $p = 0.010$; Mann-Whitney U-test $Z = 2.38$, $p = 0.015$) and parasitoid taxa ($t = 2.27$, $p = 0.033$; Mann-Whitney U-test $Z = 1.95$, $p = 0.053$) have lower population sizes than lower trophic level taxa, while the former two groups do not differ ($t = 1.02$, $p = 0.369$; Mann-Whitney-U-test $Z = 0.856$, $p = 0.476$; Fig. 1b).

To test whether our results obtained above are robust, we performed a number of alternative analyses. First, instead of the simple ANOVA presented above, we performed an ANOVA using the whole pasture by taxonomic group

data matrix with taxa nested in trophic group and pasture, and taxa as a random effect. Second, we lumped all the rare taxa excluded above into one additional, "waste basket" taxon, thus obtaining one additional taxon for each trophic group. Finally, above we considered the Phoridae to be saprophagous, although some of them are possibly parasitoids; we therefore repeated the analyses treating the Phoridae as parasitoids. In all cases, the results were qualitatively similar to those reported above.

DISCUSSION

Our data show that (behavioural) attraction to the dung was not affected by ivermectin (Fig. 2, chapter 3). We therefore conclude that reductions in the emergence data are in the majority of cases due to mortality caused by direct or indirect effects of ivermectin rather than to attractance or avoidance.

The top trophic level was significantly more reduced in dung treated with ivermectin than the lower trophic level. Yet, the group exclusively responsible for this effect are the parasitoid Hymenoptera (Fig. 1a). Predators are not more affected by ivermectin than the lower trophic levels. Thus, although our study does confirm the general hypothesis that top trophic level organisms are particularly susceptible to disturbance, this statement here needs to be restricted to parasitoids but not predators.

To explore the reasons for the observed patterns, we estimated the population sizes for the three trophic groups by considering average numbers of adult insects emerged from untreated dung only, reflecting the undisturbed ecosystem. As expected, top trophic level taxa (predators and parasitoids) had much smaller population sizes than lower trophic level taxa (Fig. 1b). This remains when analyzing predators and parasitoids separately. Parasitoids therefore fulfil our theoretical predictions: their numbers are low and the ivermectin-induced reduction of this group is significantly stronger than that of lower trophic levels. However, for predators this explanation does not hold: although their numbers are low, perhaps even lower than those of parasitoids, they are not more sensitive to ivermectin than the lower trophic levels. As parasitoids are likely to be smaller than predators on average, large body size *per se* may mediate rarity but apparently does not mediate sensitivity to pharmaceuticals in our case here (cf. Pimm 1980; Cohen *et al.* 1993, 2003).

Another important factor that can be responsible for the increased sensitivity of the top trophic level to chemicals is biomagnification (e.g. Connell 1990). Ivermectin is strongly lipophilic and thus possesses a key feature of biomagnifying substances, yet it degrades relatively rapidly (Reinemeyer & Courtney 2001), persistency being another characteristic of biomagnifying chemicals. A study with three mammalian species (Chiu *et al.* 1990) found little accumulation of ivermectin in the fat tissues, and no significant accumulation occurred in the earthworm *Eisenia fetida* (Sun *et al.* 2005). The half-life of ivermectin in cattle, pigs and dogs ranges between 2 and 8 days (Lo 1985). We therefore conclude that the biomagnifying potential of ivermectin is limited, so that the increased sensitivity of parasitoids to ivermectin is unlikely explained by this mechanism.

An alternative, indirect explanation for the strong impact on parasitoids is that the population sizes of their host taxa might have been depressed by ivermectin. This is probably not the case for the predators in the dung community, as they are not more affected by ivermectin than their potential prey. Unlike predators, parasitoids are less generalist and consequently would suffer if their hosts were disproportionately affected by ivermectin. However, hosts weakened by pharmaceuticals may also actually benefit predators and parasitoids. Delpuech *et al.* (1996) found that the immune reaction of *Drosophila melanogaster* to a parasitoid attack was suppressed by insecticides, and Floate & Fox (1999) observed increased numbers of parasitoids from a host that had been treated with a low concentration of ivermectin (similar to the one used in this study), while higher concentrations led to reduced survival in the parasitoid. Yet, as we do not know the specific relationships of parasitoids and hosts in the cattle dung ecosystem, we can neither refute nor accept the hypothesis that reduced host populations indirectly led to the reduced emergence of parasitoids in our study.

A last remaining potential mechanism to explain our results would be an extreme phylogenetically inherent sensitivity of all Hymenoptera to ivermectin, e.g. mediated by some fundamental taxon-specific physiological property. Our finding that parasitoid Hymenoptera are among the taxa to be reduced most by pharmaceutical residues is supported by the field studies of Floate (1998) and Schmidt (1983) for ivermectin and Floate (2002) for two

other avermectins. In another study, where a whitefly species was exposed to 14 insecticides, Price & Schuster (1991) found that only one insecticide did not significantly reduce parasitoids of this species. This latter result would rather support the hypothesis that the trophic position is responsible for parasitoid sensitivity, as the physiological mechanisms for these 14 insecticides are probably very different. Again, we cannot exclude a phylogenetic effect on sensitivity; to do so we would need more comparative data on hymenopteran vs. non-hymenopteran sensitivity to pollutants.

In summary, our study shows that although the top trophic level seems to be most sensitive at first glance, when having a closer look this hypothesis is not generally supported because the effect is entirely driven by hymenopteran parasitoids. This situation is fundamentally different from other ecosystems, in which often only predators (primarily vertebrates) account for the higher sensitivity of the top trophic level (Pimm 1980, 1982; Cohen *et al.* 1993, 2003). An important implication of our findings is that the assessment of any unintentional effects of chemical residues on non-target organisms should include different trophic levels. So far, typically single species of lower trophic levels are used for such assessments, simply because they are more easily bred in the laboratory (e.g. Römbke *et al.* 2009, 2010). Our study further shows that ivermectin inadvertently excreted into the environment can profoundly disturb the composition of the cattle dung insect community at the landscape scale. Although the precise causes and consequences of such selective impact are thus far unknown, man-made livestock medications likely affect the function and ecosystem services of the dung community as a whole (i.e. the decomposition of dung: Sommer & Bibby 2002).

ACKNOWLEDGMENTS

We thank the Velux foundation for generously financing this PhD project. We also thank Thomas Walter, Agroscope Reckenholz-Tänikon (CH), for supplying access to and data on the pastures studied during this project.

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Table 1: Larval feeding habits of the taxa found in cattle dung in this study as a basis for trophic classification. The last column indicates taxa excluded from analysis due to low numbers.

taxon	feeding	reference	included?
Anthomyiidae	saprophagous	Ferrar 1987	yes
Aphelinidae	parasitoid	Skidmore 1991	no
Azelia Robineau-Desvoidy, 1830	predator	Skidmore 1985	yes
Braconidae	parasitoid	Skidmore 1991	yes
Ceraphronidae	parasitoid	Skidmore 1991	no
Coenosia Meigen, 1826	obligate predator	Skidmore 1985	no
Culicoides Latreille, 1809	bacterivorous, nematophagous	Mullen & Hribar 1988	yes
Cynipoidea	parasitoid	Skidmore 1991	yes
Dasyphora Robineau-Desvoidy, 1830	coprophagous	Skidmore 1985	yes
Diapriidae	parasitoid	Skidmore 1991	yes
Dolichopodidae	predator	Robinson & Vockeroth 1981	no
Drapetis Meigen, 1822	predator	Papp 1971	yes
Drosophila Fallén, 1823	saprophagous	Wheeler 1987	no
Fannia Robineau-Desvoidy, 1830	saprophagous	Pont 2000	no
Forcipomyia Meigen, 1818	mycophagous	Mullen & Hribar 1988	yes
Haematobosca stimulans (Meigen, 1824)	coprophagous	Skidmore 1985	yes
Hebecnema Schnabl, 1889	obligate predator	Hammer 1941, Skidmore 1985	yes
Hydrotaea albipuncta (Zetterstedt, 1845)	obligate predator	Hammer 1941, Skidmore 1985, 1991	yes
Hydrotaea cinerea Robineau-Desvoidy, 1830	predator	Skidmore 1991	no
Hydrotaea irritans (Fallén, 1823)	predator	Skidmore 1991	no
Hydrotaea meridionalis Portschinsky, 1882	predator	Skidmore 1991	no
Ichneumonidae	parasitoid	Skidmore 1991	yes
Lestremiinae	mycophagous	Gagné 1981	yes
Limoniidae	saprophagous	Lindner 1959	yes
Mesembrina meridiana (Linnaeus, 1758)	facultative predator	Skidmore 1991, Muirhead Thomson 1937	yes
Morellia Robineau-Desvoidy, 1830	coprophagous	Skidmore 1985	yes
Musca autumnalis De Geer, 1776	coprophagous	Skidmore 1985	yes
Mydaea Robineau-Desvoidy, 1830	predator	Skidmore 1991	yes

Table 1 continued

Myospila meditabunda (Fabricius, 1781)	obligate predator	Hammer 1941, Skidmore 1985, 1991, Muirhead Thomson 1937	yes
Neomyia Walker, 1859	coprophagous	Skidmore 1985, Muirhead Thomson 1937	yes
Ocydromia Meigen 1820	predator	Chvala 1983	no
Orthocladiinae	saprophagous	Oliver 1971	yes
Phoridae	saprophagous, parasitoid	Disney 1994	yes
Platygastridae	parasitoid	Skidmore 1991	no
Proctotrupidae	parasitoid	Skidmore 1991	no
Psychodidae	coprophagous	Valiela 1974	yes
Pteromalidae	parasitoid	Skidmore 1991	no
Scathophaga lutaria (Fabricius, 1794)	coprophagous	Hackman 1956	yes
Scathophaga stercoraria Meigen, 1803	coprophagous	Blanckenhorn et al. 2010	yes
Scatopsidae	saprophagous	Haenni & Vaillant 1994	yes
Scelionidae	parasitoid	Skidmore 1991	no
Sciaridae	mycophagous	Skidmore 1991	yes
Sepsidae	coprophagous	Pont & Meier 2002	yes
Sphaeroceridae	coprophagous	Valiela 1974	yes
Sylvicola Harris, 1776	saprophagous	Peterson 1981	yes
Syrphidae (*Eristalis*, *Rhingia*)	saprophagous	Thompson & Rotheray 1998	no

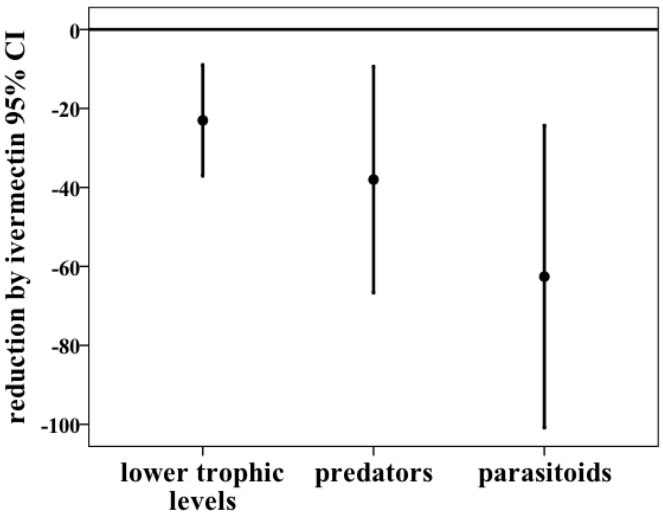

Fig 1a: Mean ± 95% CI % reduction (%) in ivermectin-treated vs. untreated control dung of emerging adults from 30 taxa grouped into three trophic levels. The number of individuals for each taxon emerging from control dung was subtracted from the number of individuals emerging from ivermectin-treated dung and divided by their sum.

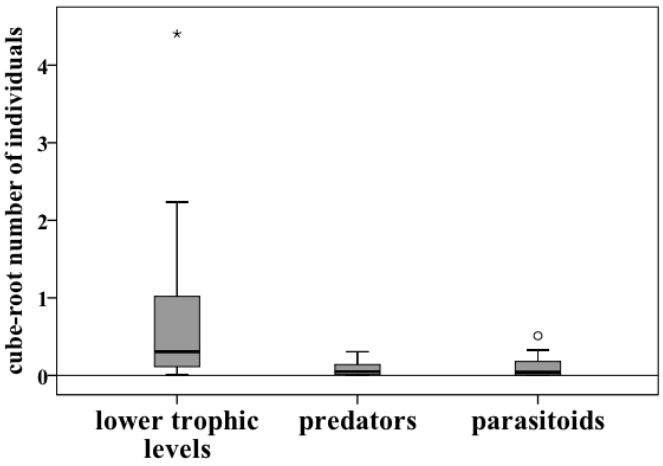

Fig 1b: Population size ranges for the trophic groups from Fig 1a. Lines inside boxes represent medians. The asterisk denotes an outlier more than 3.5 SE from mean, the circle is an outlier between 2.5 and 3.5 SE from mean, apart from that whiskers cover the whole range of values.

A field test of the effect of varying ivermectin concentrations on the biodiversity of cattle dung insects

R. Jochmann and W. U. Blanckenhorn

ABSTRACT

Veterinary pharmaceutical residues can cause severe damage in the dung ecosystem. Depending on the way of application and the time after treatment, the excreted concentration of a given pharmaceutical varies, so its effect on the organisms colonizing the dung will differ accordingly. The anthelmintic ivermectin can be applied to livestock in several different ways and is excreted through the dung over a period of several days to several months with concentrations peaking and then steadily declining. In a replicated field experiment (summer and autumn), we tested six concentrations of ivermectin known to occur in the dung of treated animals plus a null control (0 - 65.67µg ivermectin/kg fresh dung) and assessed the reaction of the cattle dung insect community. As expected, overall taxa richness of the insect dung fauna emerging from spiked dung, but not the Shannon diversity index or the total number of individuals, significantly decreased with increasing ivermectin concentration. Similar responses were obtained for individual taxa (significant for Cynipoidea, Sphaeroceridae and (marginally) Braconidae; non-significant for Anthomyiidae, Sepsidae and Sylvicola spp.), whereas another six groups that were sufficiently abundant in our emergence traps clearly showed no response to ivermectin at the concentrations tested here. Because analogous pitfall trap experiments showed that ivermectin generally does not change the attractiveness of dung, these differences in emergence probably reflect differences in survival. As our sample size was limited, we generally recommend >10 (seasonal) replicates and testing higher concentrations than used here as positive controls in future such studies.

INTRODUCTION

Veterinary pharmaceuticals excreted in the dung of treated livestock can have strong effects on the dung ecosystem (e.g. Wall & Strong 1987). In general, at least some proportion of dung dwellers are negatively affected (see Floate et

al. 2005), although results vary considerably depending on the pharmaceutical and a multitude of factors, for example the way of application and the time after treatment, both of which ultimately determine the concentration excreted (e.g. Herd et al. 1996). Single species toxicological laboratory assays indicate that in addition to inducing mortality at high substance concentrations, non-lethal effects in terms of reduced growth and body size and retarded development also result at lower concentrations (e.g. Römbke et al. 2009, 2010). Dung organisms fulfill the important ecosystem service of breaking down the dung, and at the same time are part of the larger surrounding community as prey, predators, pollinators and so on. Systematic disturbance of the dung community by anthropogenic substances thus raises concerns, to the extent that regulators mandate environmental risk assessments for residues of veterinary pharmaceuticals excreted in the dung of treated livestock (Floate et al. 2005; Römbke et al. 2009; Jochmann et al. 2011). Typically, standardized single species toxicological tests of new medications (e.g. Römbke et al. 2009) are prescribed. However, it is clear that any single test species cannot capture, and hence typify, the diversity of sensitivities to any particular substance present in natural communities. In fact, typical toxicological test species, such as the yellow dung fly (Römbke et al. 2009), are likely to be common, easy to rear, and therefore probably *not* particularly sensitive to man-made pollutants, otherwise they would not be so common. Furthermore, highly controlled laboratory tests typically do not encompass the multitude of environmental factors present in the field. Therefore it would be desirable to develop a standardized field test of effects of veterinary pharmaceuticals on multiple species, ideally the entire dung community.

The toxicity to the dung community of livestock pharmaceuticals can be principally tested in two ways (Jochmann et al. 2011). Dung can be spiked with various concentrations of the substance, which is typically diluted in a solvent such as water, ethanol or acetone. This method can be standardized well and hence is typically used in toxicological laboratory tests (e.g. Römbke et al. 2009, 2010). Alternatively, excreted dung of treated animals can be used. This method is more natural but also less controllable, as the

medication can be applied to livestock in various ways (Floate et al. 2005) and is excreted over a period of several days (e.g. Lumaret et al. 1993) to months (e.g. Errouissi et al. 2001), with concentrations peaking early and then steadily declining. This method has been used in most field tests in the past (Lumaret et al. 1993, Sommer et al. 1993, Floate 1998a). Here, we used spiked dung to test six different concentrations of ivermectin known to occur in the dung of treated animals (cf. Lumaret et al. 1993, Herd et al. 1996, Alvinerie et al. 1998), plus a null control, to assess the reaction of the entire cattle dung insect community.

Ivermectin is used to eliminate parasitic nematodes, but also arthropods like ticks and lice. It is commonly applied to a large number of livestock species worldwide. Excretion occurs through the feces. We expected that, on average, higher ivermectin concentrations would result in stronger reductions of the total number of taxa (taxa richness), individual numbers, and hence the biodiversity of the dung community, and that most taxa would show some sort of negative dose response to ivermectin. We accompanied this appraisal of insects emerging from (i.e. breeding in) the experimental dung pats by an analogous experiment using pitfall traps. In contrast to the emergence traps, the pitfall traps capture adult insects accidentally or specifically attracted to the dung, and thus serve to assess available taxa as a control, as well as whether any particular taxa are attracted or repelled by ivermectin-spiked dung, which could bias results of the emergence traps (see Floate 1998b, 2007, Wardhaugh & Mahon 1991, Holter et al. 1993).

MATERIAL AND METHODS

We spiked well-mixed cattle dung from one farm not using livestock medication at the time of collection with seven different concentrations of ivermectin. Final concentrations were: 65.67µg ivermectin/kg fresh dung, 20.75µg ivermectin/kg dung, 6.57µg ivermectin/kg dung, 2.08µg ivermectin/kg dung, 0.657µg ivermectin/kg dung, 0.208µg ivermectin/kg dung, and 0µg ivermectin/kg dung (null control of solvent only). The ivermectin was dissolved in acetone, and for each replicate dung pat 1ml of this solution was applied to 600g of dung, which was subsequently placed on five liters of soil contained in

a plastic bowl. The bottom of the bowl was perforated to allow water to drain while preventing organisms from escaping. The bowl was placed into the ground with its rim level with the surface. Every concentration was replicated three times, so that 21 dung pats were laid out in total. The replicates were randomly distributed on an experimental pasture. This setup was carried out in summer and repeated in autumn 2009 to obtain a total of 2x3 = 6 seasonal replicates.

After one week in the field, the dung pats were transferred into an emergence box. This box had only one exit into an ethanol-filled container. Insects developing into adults during the next 6 (summer) or 24 weeks (autumn) were captured in these containers. The prolonged capture period in autumn served to additionally obtain those taxa hibernating in the dung. All captured insects were subsequently identified. However, because several beetle species have long life cycles and thus probably were only represented in a biased way in our samples, we excluded Coleoptera from our analysis here. Per pat we computed three measures describing biodiversity of a community, the number of taxa (i.e. taxa richness), number of individuals (abundance), and the Shannon index of diversity, which combines information about numbers and abundance (Pielou 1974). Note that the taxonomic levels of the identified groups differ, some being species, others subsuming genera or even families; however, this should not introduce systematic bias.

The three diversity measures were analyzed using regression with ivermectin concentration as a continuous variable and season as an additional factor. We further analyzed the response of every taxon (emerged numbers only) to increasing ivermectin concentration in the same way. Of a total of 47 taxa, only those that emerged from at least half of the traps with the lowest three (i.e. benign) ivermectin concentrations (<1µg/ kg dung: 0µg, 0.208µg & 0.657µg) were deemed common enough for this analysis. Ten of 12 correlations are negative, of which however only two (three) are significant.

We performed an analogous experiment using dung-baited pitfall traps of the same ivermectin concentrations in the summer of the years 2008 and 2009. In contrast to the emergence traps, these traps captured accidental visitors or adult insects specifically attracted to the dung. This served (1) as a reference of which insects were available on the pasture at the time of the experiment and which may, for some reason or another, not have been captured in the emergence traps, and (2) to specifically test if particular taxa were attracted or repelled by ivermectin-spiked dung, which would bias results of the emergence traps (Floate 2007, Holter et al. 1993, Wardhaugh & Mahon 1991). The pitfall traps caught more beetles, many of which may not have emerged from the emergence traps due to long life cycles, but probably fewer flies, so the taxonomic composition of the two trap types was not expected to be identical.

RESULTS

Emergence traps

ANCOVA with season as a fixed factor and ivermectin concentration as a continuous covariate revealed the expected decrease with higher ivermectin concentrations only for the number of taxa (i.e. taxa richness; ivermectin effect: $F_{1,38}$=17.15, P<0.001) (Fig. 1). Taxa richness did not differ between the seasons ($F_{1,38}$=0.001, P=0.982); the interaction was also not significant ($F_{1,38}$=2.02, P=0.163), indicating equal ivermectin effects in both seasons, and was hence dropped from the final model. Corresponding results for the Shannon index were $F_{1,38}$=1.99, P=0.167 for the ivermectin effect, a significantly lower taxonomic diversity in autumn (season effect: $F_{1,38}$=10.24, P=0.003), with no interaction ($F_{1,38}$=1.77, P=0.192; Fig. 1). Number of individuals (log-transformed) yielded no ivermectin effect ($F_{1,38}$=0.29, P=0.597), far fewer individuals in autumn (season effect: $F_{1,38}$=91.86, P<0.001), again with no interaction ($F_{1,38}$=0.10, P=0.749; Fig. 1).

Twelve of a total of 47 taxa were common and abundant enough to yield meaningful results for the analysis of their individual sensitivity according to our criterion. Ten of 12 correlations are negative, in general indicating negative effects of ivermectin on emergence, as expected (Table 1).

However, the reduction was only significant for two (three) groups (Cynipoidea, Sphaeroceridae, (Braconidae); Fig. 2), reflecting large variation in combination with the relatively low sample size.

Pitfall traps

ANCOVA with year as a fixed factor and ivermectin concentration as a continuous covariate revealed no effect of ivermectin on the number of taxa (i.e. taxa richness; ivermectin effect: $F_{1,38}$=0.01, P=0.915). Taxa richness differed between the years ($F_{1,38}$=21.73, P<0.001), the interaction being not significant ($F_{1,38}$=0.06, P=0.817). Results for the Shannon index were $F_{1,38}$=0.19, P=0.669 for the ivermectin effect, no difference in diversity between the years (year effect: $F_{1,38}$=0.02, P=0.878), with no interaction ($F_{1,38}$=26, P=0.614). Number of individuals (log-transformed) yielded no ivermectin effect ($F_{1,38}$=0.15, P=0.701), but, like the taxa richness, a year effect ($F_{1,38}$=42.95, P<0.001), again with no interaction ($F_{1,38}$=1.04, P=0.315).

DISCUSSION

As expected, biodiversity of the insect dung fauna in our standardized field test decreased with increasing ivermectin concentration. However, this was significant only for species richness (number of taxonomic groups) and not for the Shannon biodiversity index. The latter is probably due to high (possibly random) variation among pats of the same concentration (e.g. Wall & Lee 2009) and hence reflects sample size limitations. Similarly, the response of individual taxa was significant only for Cynipoidea, Sphaeroceridae and (marginally) Braconidae, all showing the expected decrease with increasing ivermectin concentration. Three further groups (Anthomyiidae, Sepsidae, Sylvicola spp.) also showed a decrease potentially significant at higher sample sizes, whereas the other six groups that were sufficiently abundant in our emergence traps clearly showed no response to ivermectin (Table 1), at least at the concentrations tested here. We generally recommend more than six (seasonal) replicates (best >10), as well as testing higher concentrations than used here as positive controls, in future such studies.

Although this was not significant here (interactions P~0.15), Fig. 1 indicates that the effect of elevated ivermectin concentrations in reducing dung biodiversity might be stronger in summer than in autumn. This agrees with similar findings in other studies and may relate to the reduced number of taxa typically emerging from dung pats in autumn vs. spring and summer (Jochmann Chapters 3 & 4). Many taxa hibernate as larvae or pupae in or near the dung pat after colonizing the dung in autumn, while in summer they will typically emerge from the pat without diapause. We therefore expected a more balanced composition of the assessed communities when capturing insects from the autumn dung pats for a longer period of time. Yet, the winter was very mild and at least one taxon, namely the Sciaridae, apparently reproduced inside the emergence boxes, leading to huge numbers of offspring and a greater total number of individuals captured from the autumn dung pats. This could also explain the differences in the Shannon index between summer and autumn: as one taxon was extremely abundant in autumn, the Shannon index decreased.

The analysis of the pitfall traps shows that ivermectin does not change the attraction of the whole community to the dung, contrary to some such evidence in the past for some taxa (Floate 1998b, 2007, Wardhaugh & Mahon 1991, Holter et al. 1993). Therefore, we can assume that differences in emergence from different ivermectin concentrations can be attributed to differences in survival.

In conclusion, we believe that this field method is well suited to test the effect of pharmaceutical residues on the insect dung community. However, the number of replicates should be considerably increased to 10 or more, which in turn would also increase the amount of identification work. Replicating the experiment seasonally should also be considered, as results can be expected to (probably systematically) differ at least quantitatively in autumn due to winter diapause responses of many taxa.

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Table 1: Regression statistics for 12 (of 47) taxa that emerged from at least half of the traps with the lowest three ivermectin concentrations (<1µg/ kg dung: 0µg, 0.208µg & 0.657µg; season ignored). Ten of 12 correlations are negative, of which however only two (three) are significant (in bold).

Taxon	**B**	**SE (B)**	**r**	**t**	**P**	**Total emerged**
Anthomyiidae	-0.040	0.025	-0.246	-1.602	0.117	102
Azelia	-0.012	0.017	-0.108	-0.690	0.494	76
Braconidae	-0.141	0.073	-0.292	-1.939	***0.058***	228
Cecidomyiidae	-0.069	0.065	-0.167	-1.074	0.289	230
Chironomidae	-0.148	0.699	-0.033	-0.212	0.833	2909
Culicoides	-0.004	0.019	-0.035	-0.221	0.826	76
Cynipoidea	-0.026	0.012	-0.320	-2.138	**0.039**	42
Psychodidae	0.034	0.389	*0.014*	0.088	0.930	1756
Sciaridae	7.575	18.628	*0.064*	0.407	0.686	114430
Sepsidae	-0.041	0.027	-0.237	-1.540	0.131	69
Sphaeroceridae	-0.136	0.063	-0.323	-2.161	**0.037**	226
Sylvicola	-1.377	0.924	-0.229	-1.489	0.144	2927

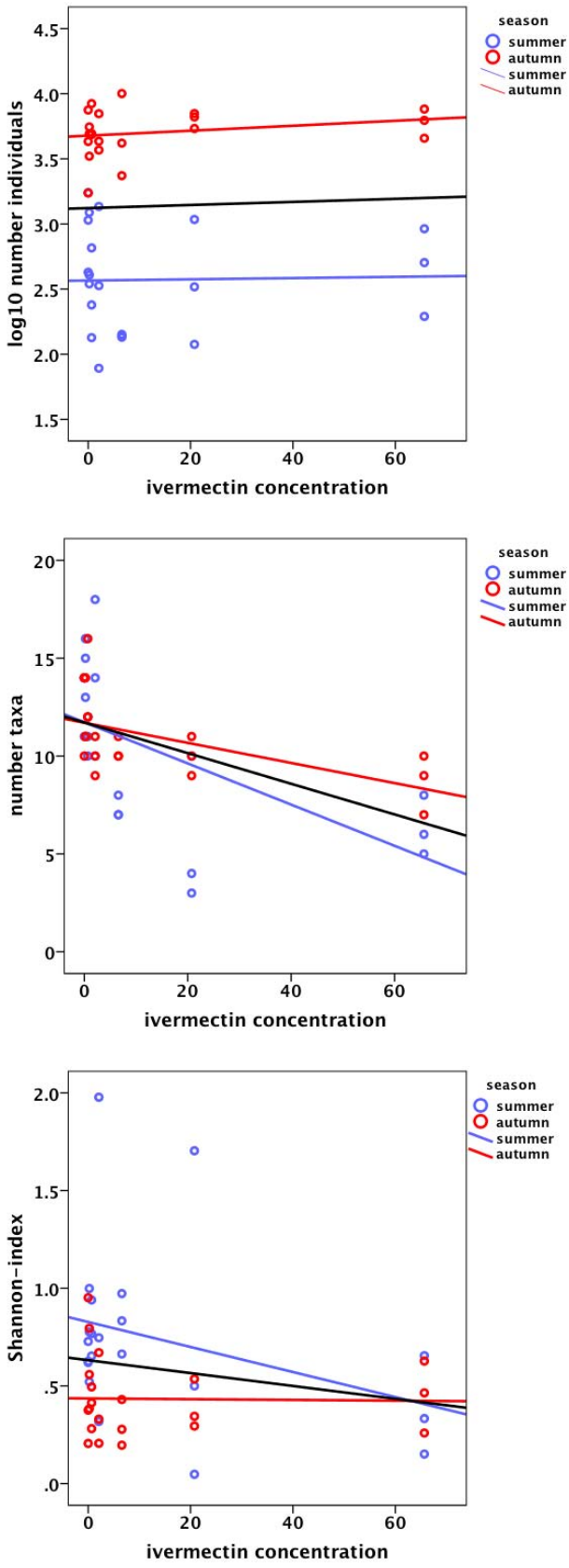

Fig. 1: Variation in number of individuals, number of taxa and Shannon index, as calculated from the taxa emerging from cattle dung, in relation to season and ivermectin concentration (in µg/kg fresh dung). The black line is the combined regression line for summer and autumn.

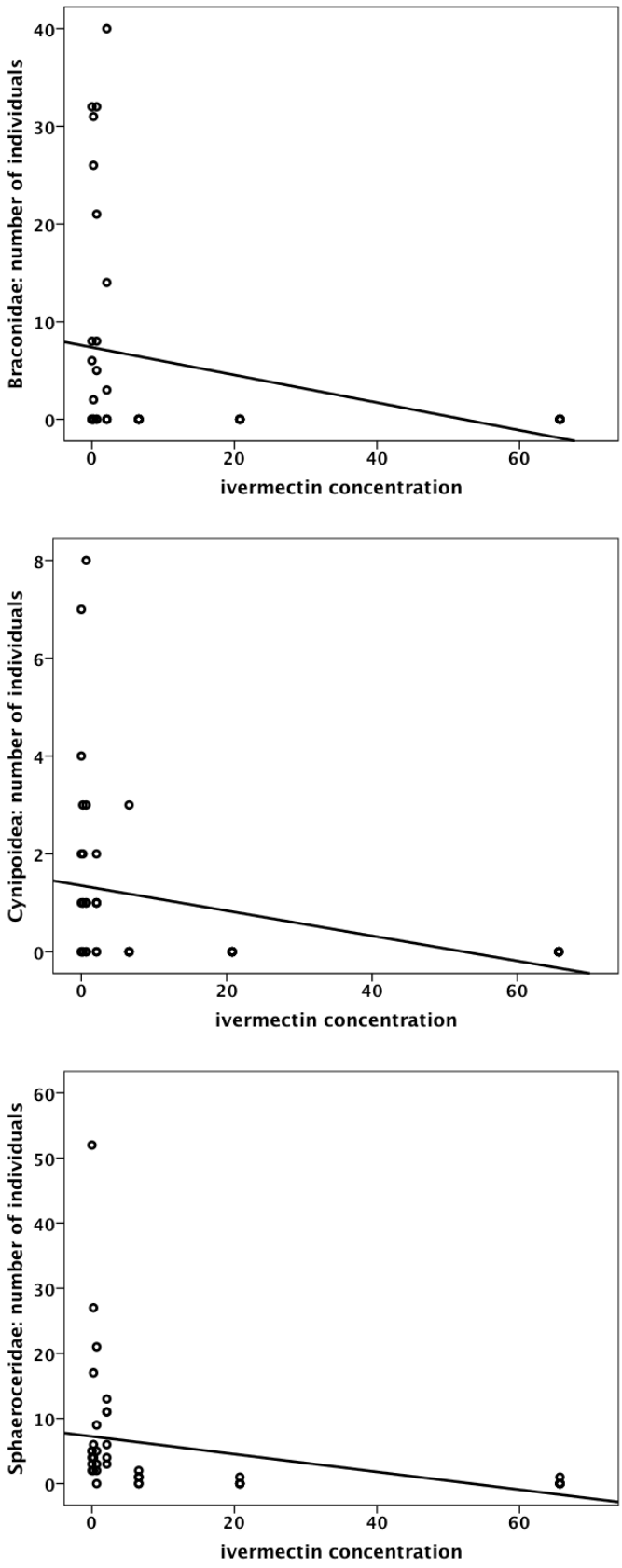

Fig. 2: Variation in number of individuals for Braconidae, Cynipoidea and Sphaeroceridae in relation to ivermectin concentration (in µg/kg fresh dung).

ZUSAMMENFASSUNG

In der Tierhaltung werden regelmässig zahlreiche Tiermedikamente in grossen Mengen angewandt. Diese Medikamente werden, verstoffwechselt oder unverändert, zumindest teilweise wieder ausgeschieden. Am stärksten von solchen Medikamentenrückständen betroffen ist somit unter anderen das Ökosystem im Dung der behandelten Tiere. Eine wichtige Voraussetzung zur objektiven und wiederholbaren Einschätzung der Folgeschäden auf das Ökosystem ist ein standardisiertes Vorgehen unter Berücksichtigung der Besonderheiten des Forschungsobjektes. Mit Kapitel 1 wird eine Vorlage hierfür geboten. Des Weiteren ist es wichtig, zwischen der Reaktion des ganzen Ökosystems und der Reaktion einzelner Arten auf einen Medikamentenrückstand zu unterscheiden. Während im Labor üblicherweise die Reaktion einzelner Arten eingehend untersucht werden kann (Kapitel 2), muss die Untersuchung der Reaktion des gesamten Ökosystems im Freiland erfolgen. Dies wurde in dieser Arbeit beispielhaft für das Medikament Ivermectin und die Gruppe der Rinderdunginsekten durchgeführt. In Kapitel 3 wird gezeigt, dass Ivermectin generell eine Reduktion der Biodiversität der betroffenen Insektengemeinschaft bewirkt. Dabei wird auch erkennbar, dass der Effekt von Ivermectin zwischen Jahreszeiten und Weiden unterschiedlich ausgeprägt sein kann. Diese Tatsache unterstreicht die Notwendigkeit, Freilandtests zu verschiedenen Jahreszeiten und an verschiedenen Orten durchzuführen. Schliesslich macht Kapitel 4 die Grenzen einer Bewertung der Medikamenteneffekte durch Laborversuche an einzelnen Arten deutlich: Ivermectin hat eine viel stärkere Wirkung auf die parasitoiden Wespen im Dung als auf die anderen trophischen Stufen. Hiervon profitieren letztere, doch dieser Effekt fehlt bei Laborversuchen mit einzelnen Arten. Praktisch alle Organismen, die bisher für Laborversuche verwendet wurden, zumindest aber alle standardmässig verwendeten Organismen, sind als Larven keine Parasitoide und keine Räuber. Während in den beiden vorhergehenden Kapiteln nur eine Konzentration von Ivermectin verwendet wurde, habe ich in Kapitel 5 sechs verschiedene Konzentrationen bewertet, welche nach Anwendung von Ivermectin üblicherweise im Rinderdung auftauchen. Eine Zunahme der Konzentration bewirkt eine Abnahme der Biodiversität, aber die Konzentrationsreihe sollte deutlich mehr als drei Mal repliziert werden.

SUMMARY

In livestock breeding, a large number of veterinary pharmaceuticals are frequently being applied in large amounts. These pharmaceuticals are at least partly excreted again, either after metabolization or unchanged. The ecosystem associated with the dung of the treated livestock is therefore among those most severely affected by these pharmaceutical residues. An important requirement for the objective and repeatable assessment of the secondary effects on the ecosystem is a standardized approach and the consideration of the particular characteristics of the study object. Chapter 1 is meant to assist as a guideline for this purpose. Furthermore, it is important to differentiate between the response of the whole ecosystem and the response of a single species to a pharmaceutical residue. While in the laboratory usually the response of single species can be examined in detail (Chapter 2), it is necessary to assess the response of the whole ecosystem in the field. This was done in this study by using the veterinary pharmaceutical ivermectin and the insects associated with cattle dung. In chapter 3 I show that ivermectin generally results in a reduction of the biodiversity of the insect community. It becomes also apparent that the effect of ivermectin varies between seasons and pastures. This emphasizes the necessity to conduct fieldwork at different seasons and localities. In addition, chapter 4 demonstrates the limitations of the assessment of the effect of pharmaceuticals by using single species in the laboratory: the effect of ivermectin on parasitoid wasps is much stronger than that on the other trophic groups, which is a benefit for the latter not present in laboratory single species bioassays. Basically all organisms used for laboratory bioassays, at least all those used as standard test organisms, are not parasitoids or predators in the larval stage. While in the two aforementioned chapters only one intermediate concentration of ivermectin was applied, in chapter 5 I assessed the effect of six different concentrations of ivermectin, all of them regularly occurring in the dung after application of ivermectin to cattle. Increasing concentrations result in a decrease of biodiversity, but the concentration series should clearly be replicated more than three times.

ACKNOWLEDGMENTS

I would like to thank

Wolf Blanckenhorn for giving me the opportunity to carry out this multifaceted and interesting project

the Velux foundation and the Zoological Museum of the University of Zurich for financing these projects

Ernst Bachofner, Peter Benz, Peter Bernet, Armin Bracher, Hanspeter Egli, Hans Elmer, Hans Greter, Christian Gyr, Andreas Hofmann, Martin Huggenberger, Hans Jud, Urs Kernen, Alois and Patrick Kohler, Meinrad Koller, Hanspeter Kühne, Marcel Küttel, Walter Looser, Thomas Ryffel, Werner Schätti, Heinrich Schiess, Jakob Schneller, Walter Schuler, Hans Schwaninger, Walter Stump, Markus Suter, Erich Unternährer and Markus Willi for letting me dig in the dung of their cattle and sometimes showing plenty of interest in what I was doing

Thomas Walter for making a lot of data available to me and thus rendering possible the whole project

Kevin Floate for an excellent collaboration

Heinz-Ulrich Reyer for being a member of my PhD-committee

Martin Schäfer, the best biologist I ever met, for reading most of my manuscripts, discussing every topic of my work exhaustively and bringing me forward enormously, all in spite of being very busy himself all the time

Sonja Sbilordo for being an excellent colleague and for combining all the Swiss qualities in one person

Ursula Briegel for fulfilling any of my wishes even before I could put them into words

Yves Choffat for a lot of support in general, and, together with Andrea Gubler, for initiating and organizing important social events

Susanne Bischof, Rosemarie Keller, Sabine Marty, Barbara Oberholzer and Isabel Schöchli, our indispensable administrative assistants, for plentiful and immediate support and for their enthusiasm for the world of animals

Alexandra Wegmann for helping me on so many occasions and, together with Angela Bahr, Maely Gauthier, Jan Knott, Stefan Sommer and Kai Stölting for discussing most of my problems with me

Oscar Ramos and Silvia Rauch for communicating in Spanish and Italian with me even when talking about science

Béatrice Nussberger, Bettina Schirrmeister and Jasmin Winkler for doing an outstanding job as PhD-representatives and for helping me on many other occasions

Christoph Germann for being a great office mate and for being one of the few with good knowledge about where to find which organisms in Switzerland

David Berger, Marco Bernasconi, Marco Demont, Richard Walters, Deborah Zulliger and Nalini for many helpful discussions

Georg Mayer for being a reliable colleague, always ready to help

Klaus Reinhold for a positive recommendation and continuous support

Michael von Tschirnhaus for introducing me to the insect world and thus creating the basis for an important prerequisite for this project

Josh van Buskirk for helpful comments on my ideas and manuscripts

Gustl Anzenberger for supporting me in many different ways

Markus Huber for the opportunity to talk about economy

Iris Biebach for being an eco-freak

Anja Ging for being a lead worker in this project

Stephanie Bauerfeind, Barbara Beck-Wörner, Anja Bürkli, Claudia Buser, Glauco Camenisch, Leyla Davis, Iris Eigenmann, Benedikt Gehr, Marta Giordano, Johann Hegelbach, Paquita Hoeck, Tracy Ivy, Priska Jud, Lukas Keller, Ursina Koller, Kerstin Musolf, Erik Postma, Nicolas Pruvost, Samuel Tanner, Barbara Tschirren, Peter Wandeler, Michèle Wegmann and Tony Wilson for contributing to a relaxed and cooperative atmosphere at the institute and thus enabling effective working

Philipp Becker, Caitlin Dmitriew, Corina Geiger, Pirmin Nietlisbach, Lena Senn and Ursina Tobler for watching real animals like birds and frogs just for fun

Christoph Bachofen, Barbara Eicher and Jeannine Roy for being excellent assistants

Marvin Brinke for advice on ivermectin related issues

Owen Petchey for giving helpful advice on one of my manuscripts

Sara Blanchard for checking the English of this section

my parents for their unlimited support

the Swiss people for hosting me in the best country of the world

ผมอยากจะมอบความขอบคุณและความประทับใจจากส่วนลึกของจิตใจผมให้กับคุณประชุมพรนวลอุไร ผู้ซึ่งเป็นบุคคลที่สำคัญที่สุดสำหรับชีวิตของผม น่าเสียดายที่ผมไม่ตระหนักถึงเรื่องนี้เป็นระยะเวลานาน เธอเป็นบุคคลที่สำคัญและมีค่าที่สุดในโลกสำหรับชีวิตของผม การที่ได้เห็นเธอมีความสุขและมีรอยยิ้มให้เป็นความสุขที่สุดของผมซึ่งช่วยให้ผมได้ทำงานนี้ได้สำเร็จด้วยดี